

The Genetic Basis of Carcinogenesis in the Clam Enterocystoplasty

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**University of London
MD Thesis
2007**



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Declaration

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Abstract.

The incidence of carcinoma following enterocystoplasty increases with time and there is an urgent requirement to develop techniques that can identify patients who will develop a life-threatening tumour.

Endoscopic biopsies were taken from the ileovesical anastomosis and native bladder remnant (control specimens) of patients who had undergone a clam ileocystoplasty. Fluorescence *in-situ* hybridisation (FISH), using centromeric probes for chromosomes 8, 9 and 18, was performed on touch sample preparations from biopsies obtained from fifteen patients. Significant aneuploid changes were found at the ileovesical anastomosis in all cases. Chromosome 18 aneuploidy was present in thirteen patients and may prove to be a useful marker of anastomotic instability.

FISH was also used to study tissue from a squamous cell clam cancer and demonstrated a large number of polyploid cells (twenty-three percent). A 'silent' p53 point mutation was identified at codon 192 by sequencing deoxyribonucleic acid (DNA) from this tumour.

The restriction site mutation (RSM) assay was used to identify rare p53 mutations in DNA extracted from biopsies obtained from thirty-eight patients. The RSM assay studied five known hotspots for mutations of the p53 gene using the restriction enzymes *Hha* I (codon 175), *Taq* I (codon 213), *Hae* III (codon 249/250) and *Msp* I (codons 148 and 282). Early p53 mutations were found at the ileovesical anastomosis at codon 213 (one patient), codon 248 (three patients) and codon 250

(three patients). The mutations were characterised by sequencing the undigested, mutated polymerase chain reaction products obtained by RSM analysis.

The anastomosis of patients who have undergone a clam ileocystoplasty is inherently genetically unstable and therefore prone to cancer formation. Both FISH and the RSM assay show promise as screening techniques and may prove to be useful in identifying those patients most at risk of developing a life-threatening tumour.

Acknowledgements.

This work was carried out between the Department of Urology at the University Hospital of Wales in Cardiff and the Centre for Molecular Genetics and Toxicology in the School of Biological Sciences at the University of Wales in Swansea.

I would like to express my sincere gratitude to Doctor Spencer Jenkins for all of his advice and encouragement in preparing this thesis and to Professor Jim Parry for his sound advice and for overseeing this project.

I am greatly appreciative to Doctor PJR Shah for acting as my London supervisor, Professor Howard Kynaston for acting as my local supervisor and to Doctor Timothy Stephenson for providing me with opportunity to undertake this project and obtaining the clinical specimens.

I am indebted to Doctor Liz Parry for her encouragement and advice and am extremely grateful to Doctor Shareen Doak and Doctor Gareth Jenkins for their patience, help and advice concerning the experimental techniques.

I am grateful to Mr Timson Appanna for donating DNA from specimens used in previous research into genetic abnormalities in clam ileocystoplasties.

I would also like to thank Di Elwell, Sally James and Sue Fielding for all their help, advice and technical assistance throughout the duration of my studies and Caroline Andrews for her secretarial support. I am grateful to Dave Pullen for Japanese translation and to Azad Hawizy for providing statistical analysis.

Finally, I thank all the patients of the University Hospital of Wales without whom this work would not have been carried out.

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1. Introduction.

1.1. The clam enterocystoplasty.

Urinary incontinence is a common and socially embarrassing condition which considerably affects the lives of sufferers (Lieberman *et al*, 2001). The ‘gold standard’ for the diagnosis of urinary incontinence is cystometry with or without video studies (Versi *et al*, 1990) although the methodology used varies between clinicians. The difficulty in diagnosing urinary incontinence arises because although a detailed history and examination provides a framework for the diagnosis, there are often discrepancies between the patient’s symptoms and the urodynamic findings (Bates *et al*, 1973; Jarvis *et al*, 1980). Approximately thirty-five percent of symptomatic female non-neuropathic patients will exhibit involuntary bladder contractions (Cardozo and Stanton, 1980). Neuropathic patients also exhibit involuntary bladder contractions. At the University Hospital of Wales, Cardiff, over ninety percent of lifelong neuropathic patients, mainly spina bifida, exhibited involuntary bladder contractions (Barrington, 1998). Unstable contractions are evident on videourodynamic investigation in approximately sixty percent of patients with spinal injuries referred to the University Hospital of Wales (Barrington, 1998). Therefore, it is evident that an overactive bladder is a common disorder of the lower urinary tract.

The International Continence Society defines the overactive bladder syndrome as ‘urgency, with or without urge incontinence, usually with frequency and nocturia’ (Abrams *et al*, 2002). Although an overactive bladder often has a significant individual impact on the quality of life and imposes limitations on activities, most individuals with the condition do not seek medical care. The

prevalence of overactive bladder increases with age and is more common in women than men (Milsom *et al*, 2000).

An overactive bladder may be helped by bladder retraining (Holmes *et al*, 1983), biofeedback (Cardozo *et al*, 1978) and hypnotherapy (Freeman and Boxby, 1982). Neuromodulation techniques such as transcutaneous electrical nerve stimulation (Bristow *et al*, 1996), anogenital stimulation (Geirsson and Fall, 1997), sacral nerve neuromodulation (Bosch and Groen, 2000), magnetic stimulation (McFarlane *et al*, 1997) and percutaneous posterior tibial nerve stimulation (van Balken *et al*, 2001) have also shown promise in the treatment of overactive bladder. However, most patients are treated initially with anti-cholinergic drugs such as oxybutinin (Yarker *et al*, 1995), this particular drug also being effective when administered intravesically (Lose and Nørgaard, 2001). Recently, anti- muscarinic drugs which have a more specific mode of action and a greater affinity for bladder M₃ receptors have become available. Currently there are four such drugs; tolterodine (Abrams *et al*, 1998), propiverine which also has a calcium antagonist effect (Madersbacher and Mürtz, 2001), trospium chloride (Cardozo *et al*, 2000) and solifenacin (Chapple *et al*, 2004) are licensed for use in the United Kingdom. Solifenacin is longer acting and requires only once daily dosing, whereas the other three drugs are shorter acting and are taken more often. These drugs are better tolerated than oxybutinin particularly with respect to frequency and severity of dryness of the mouth. Recently, long-acting formulations of the shorter acting drugs oxybutinin (Versi *et al*, 2000) and tolterodine (Kreder *et al*, 2002) have become available. These preparations have been associated with a greater efficacy (Reinberg *et al*, 2003) and a more favourable tolerability profile, particularly in terms of the frequency and severity of a dry mouth (Rovner and Wein, 2002). Studies suggest that

transcutaneous delivery of oxybutinin may decrease the side effects of the drug whilst maintaining efficacy and transdermal patches containing oxybutinin are now widely available (Cartwright and Cardozo, 2007). The majority of patients with an overactive bladder may be substantially improved by antimuscarinic therapy. However, approximately ten percent are refractory to conservative drug treatment (Stephenson and Mundy, 1994).

Augmentation cystoplasty was originally described in the dog in 1888 (Tizzoni and Foggi, 1888) and in man in 1889 (von Miculicz, 1889). Subsequently, Yeates described the use of detubularised ileum to augment the bladder (Yeates, 1956). Detubularised bowel has since been shown to be an effective method of providing a highly compliant reservoir (Schmidbauer *et al*, 1987). Clam enterocystoplasty was seldom used until the 1950s when Couvelaire popularised the procedure for the treatment of the small, contracted tuberculous bladder (Couvelaire, 1950). The clam enterocystoplasty was subsequently suggested as a treatment of lifelong day and night enuresis secondary to detrusor instability (Bramble, 1982). The operation is most commonly used for the management of detrusor instability of unknown cause, primarily in middle-aged females but is also performed in males (Mundy and Stephenson, 1985). The development of a reliable artificial urinary sphincter has also allowed the reconstruction of most motivated ambulant patients with neuropathic bladders (Stephenson and Mundy, 1985).

To perform a clam enterocystoplasty (Figure 1.1) the bladder is approached through a lower midline or Pfannenstiel incision. The bladder is incised coronally so that it opens up like a ‘clam shell’ and the incision extended well in front of the ureteric orifices to within a centimetre of the bladder neck to facilitate efficient

Figure 1.1. Operative procedure for augmentation 'clam' ileocystoplasty (Stephenson, 1988; Mundy, 1990; Nurse and Mundy, 1993).

Figure 1.1a. The bladder is incised coronally so that it opens up like the 'shell of a clam'.

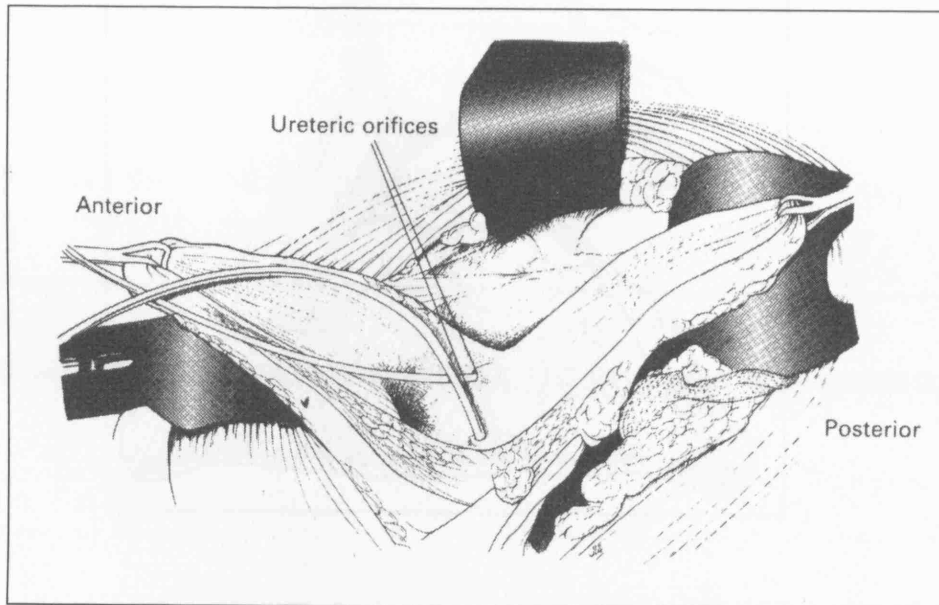


Figure 1.1b. A length of ileum equivalent to the circumference of bladder at least two feet from ileocaecal valve is identified and the mesentery inspected to ensure that the arterial supply is adequate.

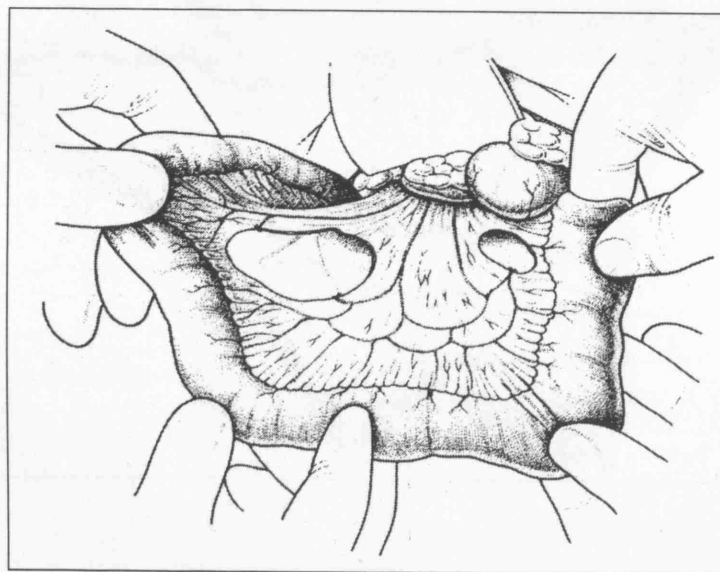


Figure 1.1c. The section of ileum is isolated on its vascular pedicle and the continuity of the divided ileum returned by an end-to-end anastomosis.

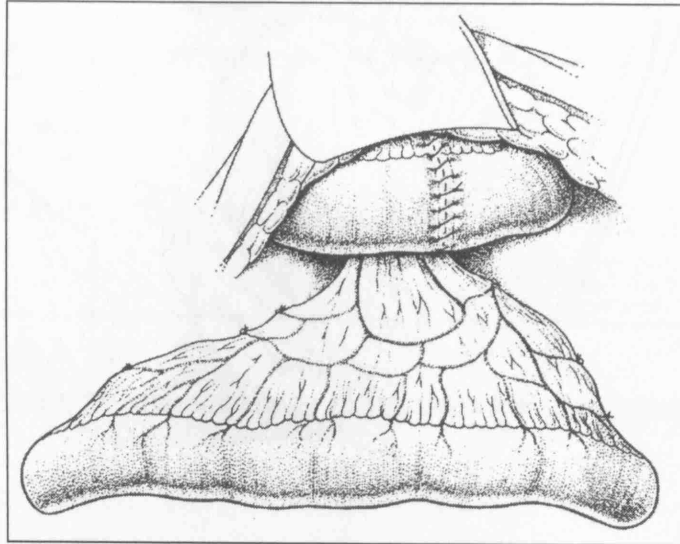


Figure 1.1d. The ileum is detubularised along its antimesenteric border.

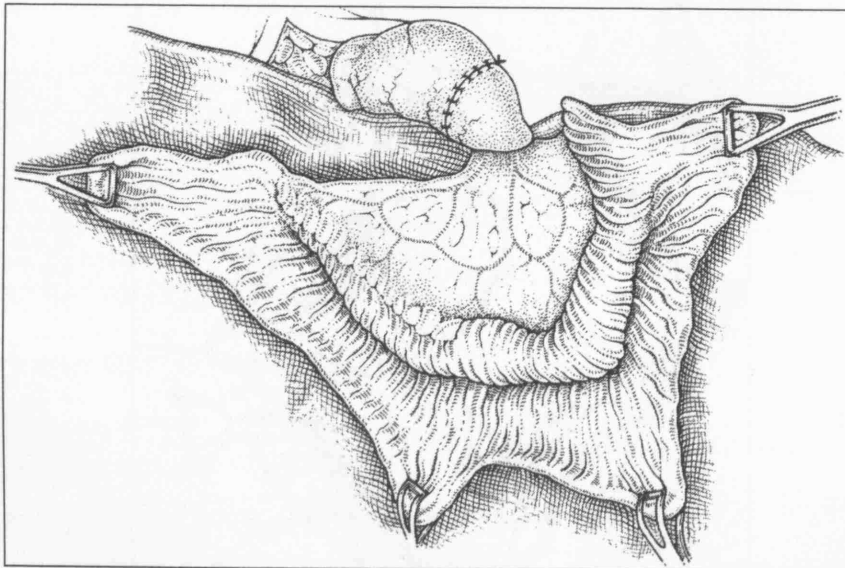


Figure 1.1e. The ileum is anastomosed to the posterior margin of bladder.

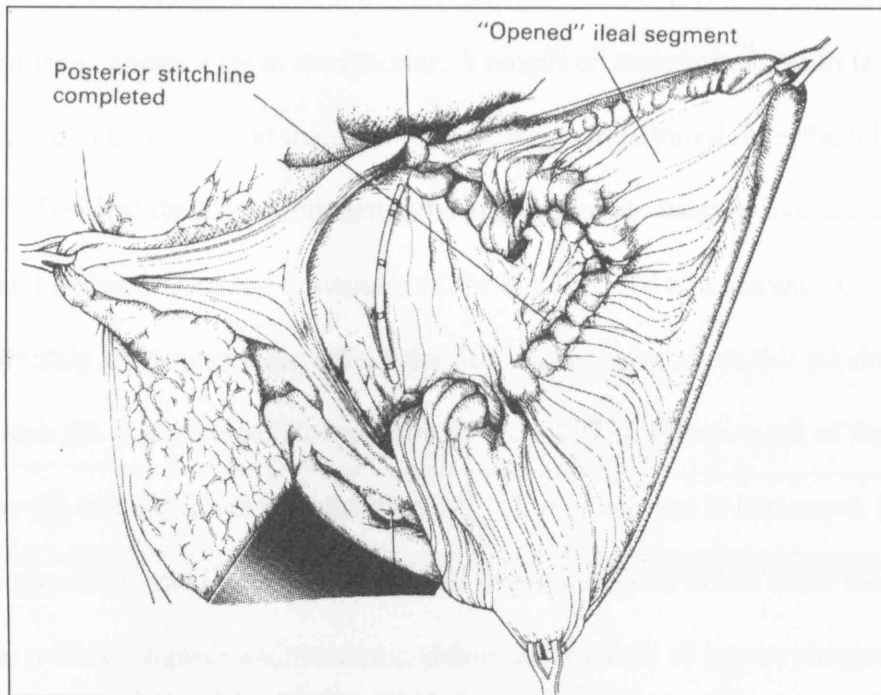
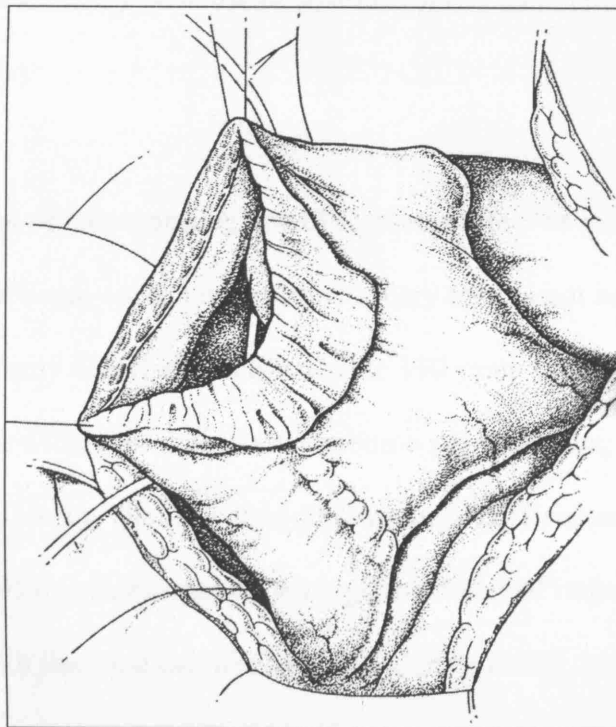


Figure 1.1f. The bladder augmentation is completed by anastomosing the ileal patch to the anterior margin of bladder.



bivalving of the bladder and to prevent the development of a pseudo-diverticulum. The ileum is assessed to ensure that the length of mesentery is sufficient to allow for a tension-free anastomosis to the bladder. A length of preterminal ileum is isolated on its own blood supply and the detubularised segment sutured onto the 'clammed' bladder. The ideal ileal segment should be approximately twenty-five centimetres in length and isolated from the remainder of the small bowel at a distance of approximately forty centimetres from the ileo-caecal valve since this produces the least metabolic disturbance (Hofmann and Poley, 1972). If the length of ileal mesentery is not sufficient then the sigmoid colon or caecum is harvested. However, wherever possible the ileum is always used in preference to colon since it is less likely to produce significant metabolic abnormalities such as hyperchloraemic acidosis and hypokalaemia (Wagstaff *et al*, 1991). In older women the mesenteric pedicle is brought anteriorly to the broad ligament whereas in the young female the pedicle is passed through the broad ligament to reduce the risk of potential damage to the blood supply should any obstetric or gynaecological surgery be necessary (George *et al*, 1991).

The concept of incorporating bowel segments either as isolated segments for diversion or in functional continuity into the urinary tract is not new. Ureterosigmoidostomy was first described some 150 years ago (Simon, 1852) although it was not widely used until after submucosal tunnelling of the ureters was described (Coffey, 1911). The ileal conduit introduced by Bricker in 1950 (Bricker and Eiseman, 1950) was associated with a high incidence of impaired renal function in patients with both ileal and colonic conduits. Consequently, urinary diversion in the young patient was uncommon and bladder reconstruction the preferred option. The success rate of a clam ileocystoplasty combined with the insertion of an artificial

sphincter in achieving continence and protection of the upper tracts in patients with neuropathic bladders is ninety percent (Venn and Mundy, 1998). An equally high continence rate (ninety-three percent) is achieved in patients with a non-neuropathic bladder who undergo a clam enterocystoplasty procedure (Venn and Mundy, 1998).

Post-operative video-urodynamic studies suggest that a number of factors contribute to the symptomatic improvement following a clam enterocystoplasty. Bivalving the bladder splits the circular continuity of the detrusor fibres and converts a spherical organ into two flat planes which either abolishes unstable activity or makes any residual contractility less effective (Mundy and Stephenson, 1985). Secondly, the interposition of an intestinal segment, which has been detubularised to abolish its own contractile activity, tends to absorb any residual detrusor contractility. Finally, of lesser importance, the volume of the bladder is increased, although this may be of greater significance in pathologically small neuropathic bladders. Not surprisingly, voluntary voiding contractions are also often affected as a result of a relative outflow obstruction due to the low-pressure cystoplasty. Thus, unless surgical manipulation of the sphincter mechanism has been carried out using sphincterotomy and the insertion of an artificial urinary sphincter (Stephenson and Mundy, 1985) approximately twenty-five percent of female non-neuropathic bladder patients and seventy-five percent of neuropathic patients (Barrington, 1998) have to void by clean intermittent self-catheterisation (Lapides *et al*, 1972). It is imperative that the common requirement for lifelong clean intermittent self-catheterisation is accepted by the patient prior to surgery and should be regarded as a consequence and not a complication of the procedure. Some may regard the need for clean intermittent self-catheterisation as a failure of the operation but the previously incontinent, but now dry patient is usually pleased with the result (Bramble, 1990).

Clam enterocystoplasty is accompanied by a substantial morbidity. The need to self-catheterise has already been mentioned. Mucus production from the intestinal segment can be substantial and averages thirty-seven grams per day (Murray *et al*, 1987). This is not usually a problem in patients who void spontaneously but can cause problems in those with catheter blockage. Mucus accumulation can predispose to urinary tract infections, stone formation and bladder outflow obstruction, particularly in patients with neuropathy (Rushton *et al*, 1988). Oral ranitidine (George *et al*, 1992) or pentosan polysulphate (Barrington *et al*, 1996a) can reduce the amount of mucus produced and acetylcysteine washouts help to dissolve excess mucus (Gillon and Mundy, 1989).

Asymptomatic bacteriuria occurs in more than three quarters of all cystoplasties but the incidence of troublesome urinary tract infection (twenty percent) is much lower (Greenwell *et al*, 2001a). Large residual volumes, the presence of mucus and the need for self-catheterisation are all predisposing factors to clinically relevant urinary tract infections.

Stone formation has been reported in as many as twenty percent of patients following enterocystoplasty and is more common in patients with urinary stasis who need to perform self-catheterisation (Nurse *et al*, 1996). Stones are typically triple phosphate, suggesting that urease-producing bacteria may be causative (Greenwell *et al*, 2001a). Once detected, stones should be removed since they often increase in size if left and may form a nidus for urinary tract infection.

Bladder perforation is a rare, but life-threatening complication of augmentation cystoplasty with a reported mortality of twenty-three percent

(Couillard *et al*, 1993). Perforation is often spontaneous but can be precipitated by binge drinking of alcohol (Bramble, 1990) or by clean intermittent catheterisation (Elder *et al*, 1988). The diagnosis is often delayed due to a lack of awareness of the condition, the often unimpressive findings on examination and a negative radiological investigation (Bauer *et al*, 1992). Patients with an enterocystoplasty often have infected urine and therefore conservative treatment is inadequate as life-threatening peritonitis is likely. If clinical examination suggests bladder rupture the abdomen must be explored.

Bowel disturbances have been reported in up to half of all patients who have undergone augmentation cystoplasty. Troublesome diarrhoea occurs in approximately twenty-five percent of patients (N'Dow *et al*, 1998) and is partly due to disruption of the enterohepatic circulation (Barrington *et al*, 1995). In our own practice some patients have shown improved bowel function with regular use of oral colestipol. Sigmoid cystoplasty (N'Dow *et al*, 1998) appears to be associated with less bowel disturbance (6.3 percent) than ileocystoplasty (fifty-five percent).

Hyperchloraemic acidosis commonly occurs following augmentation cystoplasty due to the resorption of ammonia and to a lesser extent, secretion of bicarbonate by the bowel segment. However, this metabolic abnormality is seldom of significance except in children where chronic acidosis can result in metabolic bone disease and may require bicarbonate prophylaxis (Nurse and Mundy, 1989a). Colocystoplasties are also occasionally associated with hypokalaemia due to secretion of potassium by the colonic segment (Wagstaff *et al*, 1991).

Deterioration in renal function has been reported in up to fifteen percent of patients after augmentation cystoplasty and is related to baseline renal function. Patients with a lower postoperative creatinine clearance are more likely to experience deterioration in renal function after an augmentation cystoplasty than those with normal creatinine clearance (Greenwell *et al*, 2001a). However, most patients have a stable renal function after enterocystoplasty and functional improvement has been noted in up to four percent (Khoury and Webster, 1990). In our own practice, patients are followed up with six monthly urea and electrolyte measurements and yearly ultrasound of the genitourinary tract after operation in order to monitor any alteration in renal morphology and to detect any stone formation. However, in some centres patients are reviewed yearly with ultrasound and urea and electrolyte measurements (Venn and Mundy, 1998). It has been shown that with regular monitoring, the incidence of all complications following enterocystoplasty can be kept to a minimum (Venn and Mundy, 1998). Despite the frequency of complications there is a high level of patient satisfaction in those who have undergone the procedure and the operation is often well-tolerated (Herschorn and Hewitt, 1998). There is however, mounting evidence that enterocystoplasties have an inherent potential for carcinoma formation and this remains a lasting concern.

1.2. Mechanisms of carcinogenesis.

1.2.1. The multistep nature of carcinogenesis.

Cancer is a major cause of morbidity and mortality and results from the malfunctioning of one of the many cells that make up the body. The term cancer encompasses more than one hundred forms of the disease and almost every tissue in the body is susceptible to malignancy (Weinberg, 1997). Cancer is a disease of impaired genome stability. The molecular forces which maintain genome integrity and sense altered chromosome structure are invariably subverted in cancer cells (Maser and DePinho, 2002). The exact mechanisms of carcinogenesis in humans are difficult to identify because of the long latent interval between exposure to carcinogens and recognition of tumour formation. However, the stepwise progression of human cancers is well recognised (Yokota, 2000) although it is not known precisely how many mutations are required to drive carcinogenesis

Carcinogenesis is characterised by three stages: initiation, promotion and progression (Shacter and Weitzman, 2002). Initiation occurs as the result of a carcinogen interacting with the deoxyribonucleic acid (DNA) of a cell to produce a break in the DNA or an altered nucleotide (Perantoni, 1998). Model systems have indicated that a mutation is the initiating event in carcinogenesis (Hesketh, 1997). Initiation only requires exposure to a single dose of carcinogen (Troll and Weisner, 1985) and is irreversible once a mutation is produced. Therefore, when an initiated cell divides, both daughter cells inherit the initiated state (Prescott and Flexer, 1982).

After initiation subsequent tumour progression may be mediated by further genetic alterations (Hesketh, 1997). Promoters stimulate the proliferation of initiated cells to form benign polyps or hyperplastic lesions resulting in the second stage of

carcinogenesis (Perantoni, 1998). Promotion can be achieved by exposure to the same carcinogen that gave rise to initiation or a completely different carcinogen (Prescot and Flexer, 1982). Promotion leading to tumourous growth requires multiple or prolonged exposure to a carcinogen (Troll and Wiesner, 1985).

Progression is the third stage of carcinogenesis and facilitates the conversion from benign adenomas to infiltrative and metastasising neoplasms that arise as the result of a further genetic event which give a permanent growth advantage to the initiated cell (Perantoni, 1998).

1.2.2. The clonal theory of cancer.

There is a body of considerable evidence to suggest that most neoplasms arise from a single altered cell which divides to form a neoplastic clone (Nowell, 1976). Clonal evolution within a tumour may result from enhanced genetic instability within the tumour cell population increasing the probability of further genetic changes and their subsequent selection (Nowell, 1976). It is well recognised that tumours become more aggressive in their behaviour during their clinical course, although the time course may be quite variable (Nowell, 1986). This phenomenon termed tumour progression, occurs in a stepwise fashion through qualitatively different stages (Foulds, 1957) and is reflected by the sequential appearance within the tumour of increasingly genetically altered subpopulations of cells with new characteristics (Nowell, 1986).

Tumour progression represents the results of sequential selection of variant subpopulations within a neoplastic clone. Those mutants that have an additional selective growth advantage expand to become predominant subpopulations within

the neoplasm. It is common, particularly with rapidly growing cancers, to find genetic heterogeneity within the tumour so that some components of the neoplastic population may appear much further advanced than others in their degree of tumour progression. There is also evidence that enhanced mutability increases with tumour progression (Ling *et al*, 1985) and high mutation rates are thought to play an important role in the development of metastases and drug resistance. It is probable that most new variants within a tumour cell population do not survive but those mutants that possess a selective growth advantage expand to become predominant cell populations and display the characteristics of tumour progression.

In addition to alterations within neoplastic cells, environmental factors can play a role in tumour progression by providing selective pressures which determine which mutant cells will develop into predominant subpopulations at different times (Nowell, 1986). Immunological surveillance by macrophages and natural killer cells can, by removing abnormal cells, prevent cancer but if this mechanism is impaired, tumourigenesis is more likely. Endogenous growth factors enhance the growth of certain populations of cells to grow abnormally. Several mechanisms act at the enterovesical anastomosis within an enterocystoplasty which may promote tumour formation (Chapter 1.3.3.). These processes could be particularly important in promoting the growth and development of an early neoplasm and on the continuing evolution of a neoplastic population.

1.2.3. Aneuploidy and cancer.

Aneuploidy, where a cell or organism possesses fewer or more chromosomes than an exact multiple of the haploid number is the most prevalent genetic change reported in solid tumours (Heim and Mitelman, 1995). Missing chromosomes may

result in incomplete genomic DNA and the possible loss of critically important genes whereas additional chromosomes may lead to unbalanced gene expression.

Aneuploidy is thought to be a dynamic chromosome mutation event intimately associated with cancer.

Aneuploid tumour cell lines (Lengauer *et al*, 1997) and transformed rodent cells (Duesberg *et al*, 1998) have been reported to display an elevated rate of chromosomal instability indicating that aneuploidy is a dynamic chromosome mutation event associated with transformation of cancer cells. Chromosomal instability has been shown to be proportional to the degree of aneuploidy in the transformed cells and aneuploidy is thought to be a mechanism which simultaneously alters and destabilises the normal cellular phenotype.

Aneuploid cells have been shown to develop clonal populations that can spread across an area of premalignant epithelium (Reid *et al*, 1992). In addition, it is recognised that different aneuploid cell populations can coexist together occupying defined, but sometimes overlapping spatial distributions in epithelium (Rabinovitch *et al*, 1989). DNA aneuploidy indicates a high risk of developing severe premalignant changes (Lindberg *et al*, 1999) and can be a useful indicator of lymph node metastasis (Abad *et al*, 1998). An increase in aneuploidy is associated with increasing progression and recurrence of transitional carcinoma of the bladder (Gonick *et al*, 1980; Cianiulli *et al*, 2000). However, not all studies have shown a link between aneuploidy and a worsened outcome (Ioakim-Liossi *et al*, 2000) and further work is required to clarify the precise changes which are associated with poor prognosis for a given cancer. Recently, identification of oncogenes and tumour suppressor genes, gained or lost on chromosomes in aneuploid tumours is providing

strong evidence that chromosomes involved in aneuploidy play a critical role in the tumourigenesis (Sen, 2000).

Aneuploidy is a numeric imbalance in chromosomes and is therefore thought to arise from misaggregation of chromosomes. Several possible events may lead to the production of aneuploid cells. Non-disjunction occurs when two homologous chromosomes fail to divide during mitosis resulting in one daughter cell having one less chromosome and the other having one extra chromosome. It is possible that sizeable clonal populations from each cell may be produced, or alternatively one may possess a growth advantage and predominate depending on the effect that the abnormal division has on each daughter cell. Chromosome lagging occurs if one chromosome moves more slowly than the other during anaphase and this chromosome may fail to be included in either daughter cell or may be included with its sister chromatid to produce a trisomic cell. Chromosome dislocation occurs when there is failure of attachment of a chromosome onto the metaphase spindle and the chromosome concerned becomes randomly included within daughter cells. In addition to the above mechanisms extra chromosome replication may lead to aneuploidy and chromosome loss and may result in monosomy.

Centrosomes are involved in organising the microtubule network and mitotic spindle during cell division. Multipolar mitotic spindles have been observed and supernumerary centrosomes and those with an aberrant size and shape have been described in a variety of human cancers (Pihan *et al*, 1998) Therefore, it is conceivable that cells with abnormal centrosomes may misaggregate producing aneuploid cells. Supernumerary centrosomes have been described in p53 deficient cells (Zimmerman *et al*, 1998). The centrosome-associated kinase

STK15/BTAK/aurora 2 is overexpressed in human cancers and is thought to be associated with abnormal centrosome function and abnormal segregation of chromosomes (Zhou *et al*, 1998; Bischoff *et al*, 1998).

One of the important events that ensures equal partitioning of chromosomes during mitosis is the proper and timely separation of the sister chromatids that are attached to each other and the mitotic spindle. Sister chromatid separation is inhibited by a group of proteins called securins which normally act during metaphase-anaphase transition. V-securin which inhibits sister chromatid separation and is involved in tumourigenesis has recently been identified. Therefore, over-expression of v-securin may contribute to the generation of malignant tumours resulting from chromosome gain or loss produced by errors in sister chromatid separation (Zou *et al*, 1999).

Normal progression through mitosis is monitored by two checkpoints. One checkpoint operates during early prophase (G2) to metaphase progression while the second ensures proper segregation of chromosomes during metaphase to anaphase transition. It is thought that checkpoint controls have to be overcome for unequal chromosome segregation to be perpetuated through cell proliferation cycles to produce aneuploidy. Recently, genes that regulate metaphase-anaphase transition have been identified and abrogation of function of these genes leads to multinucleation and aneuploidy (Jin *et al*, 1998).

1.2.4. Genes involved in cancer formation.

Tumour suppressor genes, proto-oncogenes and genes involved in DNA repair are the three main classes of genes involved in carcinogenesis. These genes are

essential for controlling the rate of cell division and the maintenance of genetic instability. Mutations in these genes may result in the onset of carcinogenesis, although it has been estimated that three to seven individual mutations are required for the induction of a malignant cancer (Vogelstein and Kinzler, 1993).

Proto-oncogenes regulate normal cell growth and differentiation and apoptosis (Barbacid, 1987). Over one hundred proto-oncogenes have been isolated and mapped to human chromosomes. Proto-oncogenes can be activated to oncogenes by point mutations, chromosomal translocations or gene rearrangements (Bishop, 1981). Genetic damage of a proto-oncogene can activate its expression leading to an increase in function and cellular proliferation. Activation of proto-oncogenes can free cells from their usual growth restraints resulting in uncontrolled proliferation.

Tumour suppressor genes normally prevent the development of cancer (Hooper, 1994). Over a dozen tumour suppressor genes have been characterised and localised in the genome (Perantoni, 1998). Tumour suppressor genes encode for proteins that negatively regulate cell cycle expression (Collins *et al*, 1997). Negative controls on cell cycle progression are exerted during development, differentiation, senescence and cell death (Hartwell and Kastan, 1994). Tumour suppressor genes are involved in carcinogenesis when they sustain mutations which result in a loss of function. Therefore, genetic alterations that inactivate tumour suppressor genes free the cell from the constraints imposed by these genes and result in unconstrained deregulated cell growth (Weinberg, 1991). Tumour suppressor genes are generally recessive and therefore, a mutation in one allele of a tumour suppressor gene will not disrupt its function as long as the other allele is still functional (Wijnhoven *et al*,

2001). Thus, only when both copies of the gene are activated does the cell display an abnormal phenotype (Ponder, 2001).

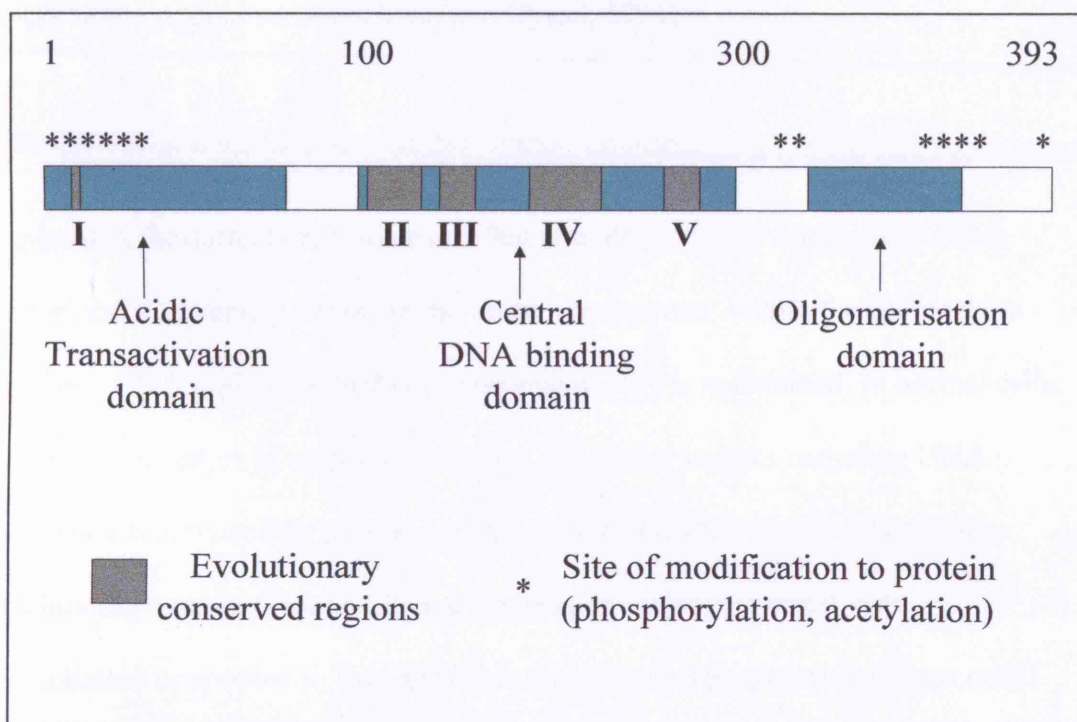
Mismatch repair genes are involved in DNA repair and in maintaining the integrity of the genome. Alterations in the functioning of these genes have been associated with an indirect role in cancer formation (Kolodner, 1995). Inactivation of mismatch repair genes allows the development and accumulation of mutations in oncogenes and tumour suppressor genes that directly affect cell proliferation. Both sporadic and hereditary colorectal carcinomas show defects in these genes, highlighting the importance of mismatch repair genes in cancer formation (Fishel and Kolodner, 1995).

1.2.5. The p53 tumour suppressor gene.

The p53 gene is located on chromosome 17p and encodes a 393 amino-acid, 53 kilodalton (kDA) nuclear phosphoprotein critical for the cellular response to genotoxic insult. P53 activation elicits cell cycle arrest or programmed cell death (apoptosis) depending on the cell type and degree of DNA damage (Keegan *et al*, 1998). Tumour suppressor genes usually promote carcinogenesis by inactivation of the respective gene and are usually recessive. P53 is a tumour suppressor gene but mutations in p53 are sometimes described as ‘dominant negative’ as one mutated copy of the gene is often enough to induce the mutant phenotype within affected cells. The p53 protein consists of a tetramer of four subunits and it is thought that a mutation in a pair of the subunits is sufficient for the tetramer to form an inactive configuration. The binding affinity of the p53 protein to DNA has been shown to be affected by the presence of mutated p53 monomers (McCure and Lee, 1998).

The p53 protein contains three main domains, an N terminal domain, a central domain and a C terminal domain (Figure 1.2). The N terminal domain contains the acidic transactivation domain which is a small regulatory region of p53 that activates transcription factors (Müller and Helin, 2000). Transcription factors are proteins that bind to DNA and control the transcription of genetic material from DNA to messenger RNA by the enzyme RNA polymerase. The C terminal domain contains the oligomerisation domain that is responsible for the oligomerisation of p53 into a four molecule tetramer which is required for the p53 molecule to adequately function (Jeffrey *et al*, 1995). The central domain contains a DNA binding domain which is capable of binding to specific DNA binding sequences present in the regulatory regions of genes which are transcriptionally regulated by p53. Two copies of the consensus sequence are required for DNA binding. The p53 gene contains five evolutionary conserved regions (that are present in a wide range of species) and four of these are present in the central (DNA) binding domain (Soussi *et al*, 1990). Most of the mutation hotspots identified in the p53 gene of human cancers are found within these four central conserved regions demonstrating the vital role that DNA binding plays in p53 function. Indeed, ninety five percent of p53 mutations detected in tumours occur in the DNA binding domain (Bullock and Fersht, 2001). It has been shown that mutations at these hotspots typically remove the ability of the consensus sequence to bind to DNA (El-Deiry *et al*, 1992). The highest concentrations of mutations within cancers are within codons 175, 196, 213, 245, 248 (the codon with the highest level of mutations), 249, 273 and 282 (Jenkins *et al*, 2001). Most mutations detected in tumour suppressor genes are nonsense mutations which terminate the translation of proteins and lead to the synthesis of truncated inactive proteins. P53 is unusual as most detected mutations are missense events which alter the amino-acids present in a protein (Harris, 1993). Most of the missense

Figure 1.2. Structure of the p53 gene (University of Wales, Swansea, unknown source).



mutations in the p53 gene are clustered within exons five to eight including those sequences which are highly conserved during evolution (Caron de Fromental and Soussi, 1992). Conversely, the amino-terminal and carboxyl-terminal domains of the p53 gene have been shown to contain more nonsense than missense mutations. Mutated p53 protein has a prolonged half-life and inactivates wild-type p53 by formation of complexes leading to uncontrolled cell growth (Brandau and Böhle, 2001). Only mutations in exons five to eight of the p53 gene result in an increased half-life of the protein product (Kroft and Oyasu, 1994).

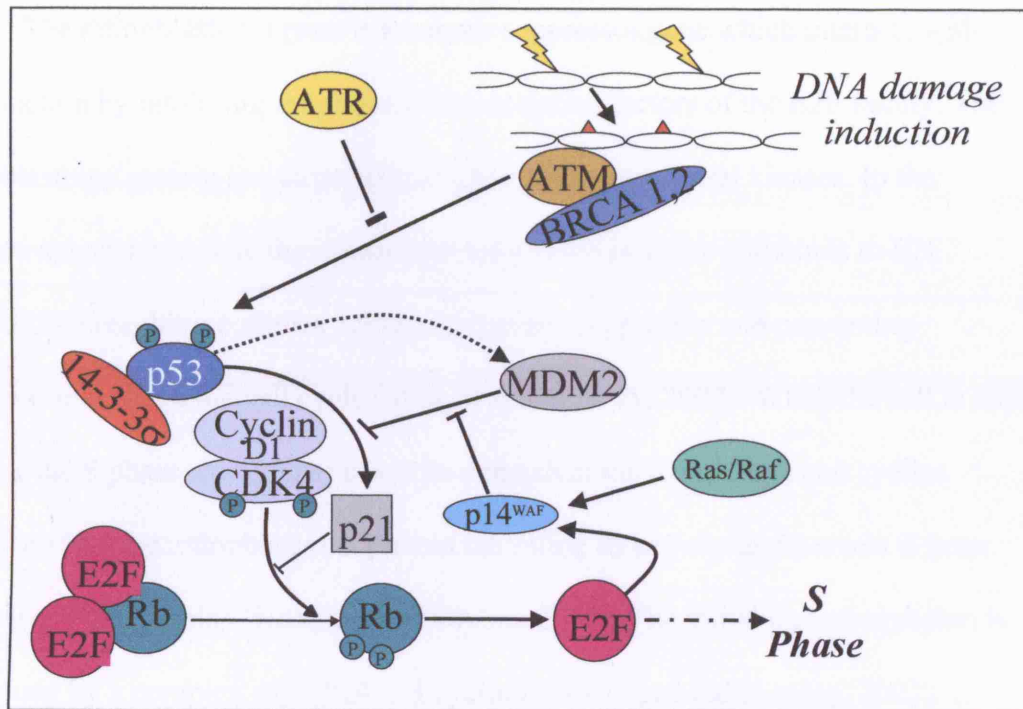
During the cell cycle there is a requirement to ensure that each stage is completed in the correct order to ensure that alterations do not occur in the DNA composition. Conversely, there are therefore ‘checkpoints’ within the cell cycle to ensure that DNA replication and chromosomal quality is maintained. In normal cells, p53 activity increases in response to a range of cellular stresses including DNA damage and is activated by phosphorylation at specific sites closely related to the DNA binding domain (Figure 1.2) and can result in cell cycle arrest, cell differentiation or apoptosis. This decreases the chance of propagating mutant cells and hence of cancer formation and p53 has therefore been described as the ‘guardian of the genome’ (Lane, 1992). Cells lacking p53, either as a consequence of p53 point mutations or a loss of a p53 allele, are free to divide uncontrollably. Some of the interactions of p53 have been well documented but relatively little is known about others. Disruption of p53 function can also be mimicked by the deletion or mutation of one of its key related effector proteins.

P53 protein is able to cause cell-cycle arrest in the G1 phase to allow repair of damaged DNA. The p53 protein migrates into the nucleus and promotes the

transcription of another protein, WAF1 (p21) (El-Deiry *et al*, 1993). WAF1 then binds and inhibits CDK2, a cyclin dependent kinase that is required for transition into the next phase of the cell cycle. A delay of this process allows DNA-repair mechanisms to function (Figure 1.3). The ability of p53 to detect damaged DNA is not fully understood but is thought to involve ATM kinase which is deficient in ionising radiation sensitive ataxia telangiectasia syndrome (Kastan *et al*, 1992). DNA damage induces the ATM gene and leads to phosphorylation of the p53 protein thus activating the p53 protein. BRCA genes also act as DNA damage sensors as part of a multisubunit complex which includes ATM (Wang *et al*, 2000) and negative feedback occurs via the ATR gene which is known to antagonise the action of ATM by inhibiting p53 phosphorylation by ATM (Abraham, 2001).

Other proteins controlling p53 activity after DNA damage include 14-3-3 σ , which enables p53 to bind to damaged DNA with a greater affinity (Waterman *et al*, 1998), and the Murine Double Minute Protein (MDM2) which negatively regulates p53 protein levels by facilitating p53 protein transport from the nucleus into the cytoplasm (Piette *et al*, 1997). Another protein, p14, allows p53 protein to accumulate after DNA damage by destabilising MDM2 (Lu *et al*, 2002). P14 production is induced by the E2F family of proteins although the precise mechanism remains obscure (Matsumura *et al*, 2003). E2F proteins are a family of transcription factors whose primary function is to push the cell into the S phase but as long as they are deactivated the cell stays in the G1 phase (Münger and Howley, 2002). Oncogenes activate the Ras and Raf family, which are another group of genes which cause G1 specific cell cycle arrest. The Ras and Raf family of genes do not affect p14 activity in normal cells but do increase p14 activity in cells containing E2F (Berkovich *et al*, 2003). Therefore, the action of E2F transcription factors in

Figure 1.3. Cell cycle arrest induced by the p53 protein (University of Wales, Swansea, unknown source).



ATM – Ataxia Telangiectasia Mutated gene product

ATR - Ataxia Telangiectasia and Rad-3 Related gene product

BRCA1, 2 – Breast Cancer 1 and 2 gene products

CDK4 – cyclin dependent kinase 4

Cyclin D1- protein initiating phosphorylation of retinoblastoma protein

E2F – family of transcription factors

MDM2 - Murine Double Minute Gene 2

P – Phosphate molecules

p14, p21, p53 – protein products of p14, p21, p53 genes

Ras/ Raf – products of Ras/ Raf family of genes

Rb – Retinoblastoma protein

14-3-3σ - protein which enhances effect of p53

activating the inhibitory effects of p14 and the Ras and Raf genes appears to antagonise and provide a negative control for the primary function of E2F transcription factors which is to initiate the S phase and synthesis of DNA.

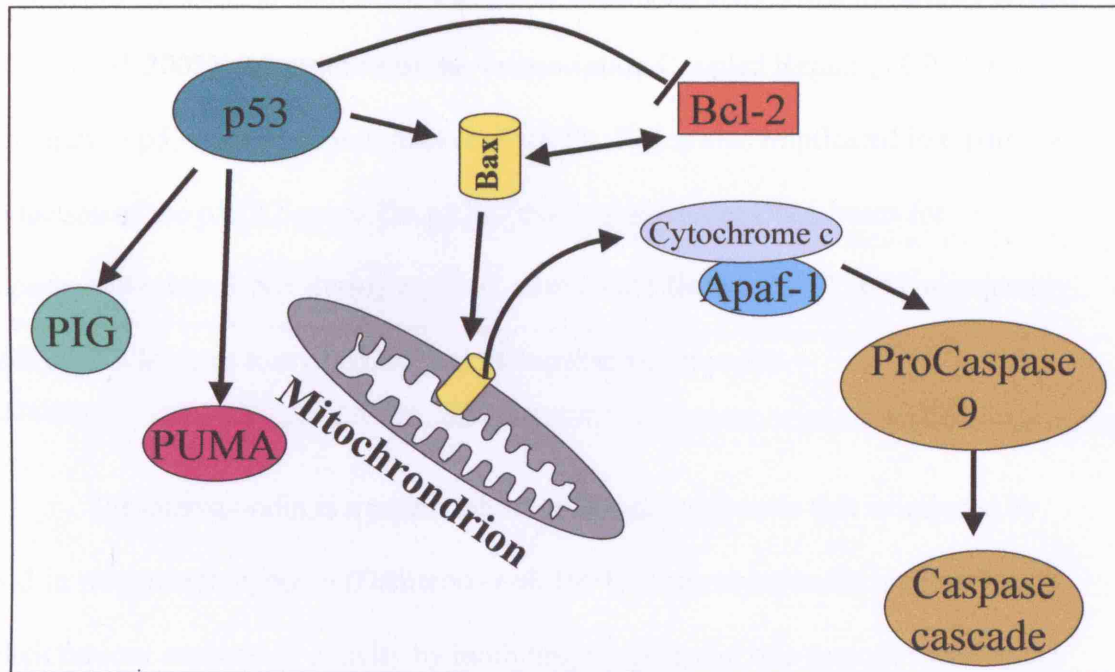
The retinoblastoma gene is a tumour suppressor gene which interacts with p53 function by inhibiting the action of transcription factors of the E2F family. The retinoblastoma protein is a target of phosphorylation by several kinases. In the hypophosphorylated state the retinoblastoma protein is active and binds to E2F proteins, the resulting complex acting as a growth suppressor and preventing progression through the cell cycle (Münger and Howley, 2002). When the cell is due to enter the S phase, complexes of cyclin dependent kinases (CDK) and cyclins phosphorylate the retinoblastoma protein inhibiting its activity and prevent it from binding to E2F proteins (Korenjak and Brehm, 2005). The initial phosphorylation is performed by a complex of CDK4 and cyclin D1 (Münger and Howley, 2002).

P53 is able to not only regulate the G1/S checkpoint but also the G2/M checkpoint via the protein GADD45 α which is induced by DNA following the detection of DNA damage (Cadwell and Zambetti, 2001). It has also been suggested that p21^{WAF} is essential for G2/M arrest (Bunz *et al*, 1998). Arrest at the G2/M checkpoint ensures the chromosomes are correctly replicated prior to entering mitosis. There is also evidence that p53 protein can cause arrest at the metaphase/anaphase transition thereby ensuring the correct alignment of chromosomes on the metaphase plate prior to segregation at anaphase (Cadwell and Zambetti, 2001).

In the presence of cell damage when DNA repair is not possible, apoptosis occurs. The apoptotic pathway prevents damaged cells which could potentially create daughter cells with considerable genetic alteration from replicating. The p53 protein interacts with proteins such as Bax (apoptosis promoting) and Bcl-2 (antiapoptotic) thereby regulating apoptosis (Figure 1.4). The ratio of Bax to Bcl-2 has been shown to determine whether the cell survives or undergoes apoptosis (Oltvai *et al*, 1993). Once activated, p53 protein increases the transcription of Bax which is inserted into the mitochondrial membrane allowing the release of cytochrome c (Suzuki *et al*, 2000). When cytochrome c is released it interacts with the cytoplasmic protein Apaf-1 to form a molecular complex which in turn activates procaspase-9 to form caspase-9 which triggers the caspase cascade. Caspases are proteases that convey the apoptotic signal by caspases activating other caspases that subsequently degrade cellular targets leading to cell death (Salveson, 2002).

However, p53 induced apoptosis can occur independently of Bax. P53 induced genes (PIGs) include apoptotic genes involved in the induction of reactive oxygen species (Polyak *et al*, 1997) which promote mitochondrial cell death (Gu *et al*, 2000). Proteins which are involved in the regulation of apoptosis by p53 include PUMA (Yu *et al*, 2001), IGFBP3, KILLER, Dr3, Fas, and Noxa (Cadwell and Zambetti, 2001). PUMA appears to be one of the most significant of these proteins in the process of apoptosis and can be directly activated by p53 (Yu *et al*, 2001) and also initiates cell death by activating bax and also by increasing the permeability of the mitochondrial membrane and subsequent leakage of cytochrome c into the cytoplasm (Vousden, 2005)

Figure 1.4. The role of p53 in apoptosis (University of Wales, Swansea, unknown source).



Apaf-1 (apoptotic protease activating factor 1) – cytoplasmic protein that forms a complex with cytochrome c that activates procaspase 9 (promotes apoptosis).

Bax – cytoplasmic protein that initiates apoptosis by inserting into the mitochondrial membrane.

Bcl-2 – protein that prevents apoptosis.

Caspase Cascade – Series of proteases that activate each other and subsequently degrade cellular targets leading to cell death.

Cytochrome c – protein released from mitochondria and forms a complex with Apaf-1 (promotes apoptosis).

PI3 (P53 induced genes) genes that promote apoptosis in response to p53.

Procaspase 9 – protein that is converted to caspase 9 which initiates the caspase cascade which results in apoptosis.

PUMA (p53 upregulated modulator of apoptosis) – protein that initiates apoptosis by several mechanisms.

The mechanisms by which p53 proteins influences DNA repair pathways are not yet fully understood. The p53 protein binds Nucleotide Excision Repair (NER) proteins (van Steeg, 2001) and therefore may regulate DNA repair activity by this process. P53 is also thought to have a role in enhancing Base Excision Repair (BER) (Zhou *et al*, 2001) and proteins of the Transcription Coupled Repair (TCR) family also induce p53 activity (Ljungman *et al*, 1999). P53 is also implicated in repair via induction of the p53R2 gene. The p53R2 enzyme produces DNA bases for incorporation into DNA during repair (Lozano and Elledge, 2000) and consequently, loss of p53 leads to loss of p53R2 and reduced repair capacity.

Thrombospondin is a potent inhibitor of tumourigenesis that is induced by p53 in response to hypoxia (Dameron *et al*, 1994). Thrombospondin is thought to elicit tumour suppressor activity by inhibiting the potential of a tumour to develop a blood supply (angiogenesis). As a tumour reaches a critical size, the blood supply becomes the rate-limiting step to further growth and the resulting hypoxia may induce p53 mediated cell death. Invasive transitional cell carcinomas with low thrombospondin expression have been shown to be associated with increased recurrence rates, decreased survival and p53 status (Grossfeld *et al*, 1997).

Mutations in the p53 gene are the most common mutation events identified in a variety of human cancers (Carson and Lois, 1995). Point mutations have been found scattered over much of the p53 gene. However, the vast majority of the mutations associated with human tumours are situated within exons five to eight of the p53 gene, the region coding the p53 protein central domain (Bérout *et al*, 1996). The frequency of p53 mutations and their distribution throughout the p53 gene (mutation spectrum) is different for individual tumour types suggesting that the

mechanisms of tumourigenesis differ for each cancer (Greenblatt *et al*, 1994). The nature of mutations present and the codons in which they occur can provide important clues to the aetiology and potential causative agents involved in the initiation and development of specific tumours (Greenblatt *et al*, 1994). The possible relationship between specific environmental exposures and the development of mutations at specific codons is currently being investigated (Lewis and Parry, 2004). The identification of such factors provides the potential for the use of molecular markers in risk reduction programmes.

In summary, the p53 gene product is critical to ensuring the completion of the cell cycle and monitoring the genetic quality of progeny cells. Such activity has led to the p53 gene product being called the ‘guardian of the genome’. The activity of the p53 gene can be modified by a variety of both point mutations and chromosomal changes which leads to the production of increased levels of genetically abnormal cells completing the cell cycle. These genetic changes in p53 can produce reduced levels of DNA repair and apoptosis. Alterations in the p53 gene are the most common mutational events described in a variety of human cancers and therefore identifying p53 mutations may be useful in highlighting those patients with premalignant conditions who will go on to develop cancer.

1.3. Carcinogenesis in enterocystoplasty.

1.3.1. Epidemiology.

There are an increasing number of reports of cancers, usually aggressive tumours with a high mortality, developing within an enterocystoplasty (Table 1.1). These tumours are mostly adenocarcinomas and are usually located at or adjacent to the enterovesical anastomosis and there is often a long latent period between the time of operation and the time of diagnosis of a neoplasm.

Tumour formation within an augmentation cystoplasty was first reported in 1971 (Smith and Hardy, 1971). Subsequently, Leedham and England described the occurrence of an adenocarcinoma within an augmentation cystoplasty (Leedham and England, 1973). In 1990 a review identified fourteen similar cases (Filmer and Spencer, 1990) and since that time the number of reported cases has more than trebled. At present sixty reports of patients with tumours within an augmentation cystoplasty have been described in the world literature (Table 1.1). Six benign tumours have also been described (Table 1.2). Forty-one of the sixty patients presenting with a tumour following augmentation cystoplasty had undergone an ileocystoplasty, sixteen patients received a bladder augmentation with large bowel and three patients had undergone a gastrocystoplasty. Six further cases of tumour within an augmentation cystoplasty are described in the literature and not included: in three cases the tumour was thought to be present at the time of operation (Lamm and Gittes, 1977; Grainger *et al*, 1988; Stillwell and Myers, 1988) and in three others the tumour was probably a recurrence of transitional cell carcinoma (Selli *et al*, 1986; Bunyaratavej *et al*, 1993; Garcia *et al*, 1993).

Table 1.1. Malignant tumours arising in bladder augmentations.

Reference	Yrs from augmentation (pt. age at tumour)	Sex	Indication	Operation	Presentation of tumour	Pathology	Site of tumour origin
Smith and Hardy, 1971.	17 (43)	female	tuberculosis	ileocystoplasty	irritative symptoms and haematuria	transitional cell carcinoma with squamous elements	ileum, near ileovesical anastomosis
Leedham and England, 1973.	15 (52)	male	tuberculosis	ileocystoplasty	haematuria	adenocarcinoma and carcinoma <i>in situ</i>	ileum near ileovesical anastomosis
Egbert <i>et al.</i> , 1980.	19 (43)	male	traumatic quadriplegia	ileocystoplasty	haematuria	leiomyosarcoma	bladder near ileovesical anastomosis
Takasaki <i>et al.</i> , 1983.	20 (42)	male	tuberculosis	ileocystoplasty	haematuria	signet ring adenocarcinoma	ileum, near ileovesical anastomosis
Kamidino <i>et al.</i> , 1985.	20 (47)	male	tuberculosis	ileocystoplasty	haematuria	adenocarcinoma	ileum
Kirby and Lloyd-Davies, 1985.	17 (31)	female	squamous carcinoma of cervix	caccocystoplasty	urethral discharge	adenocarcinoma	colon
Steg <i>et al.</i> , 1985.	21 (58)	male	tuberculosis	colocystoplasty	haematuria	adenocarcinoma	colovesical anastomosis
Harzmann <i>et al.</i> , 1986.	17 (44)	male	tuberculosis	colocystoplasty	not stated	adenocarcinoma	bladder multifocal
Stöckle <i>et al.</i> , 1986	17 (40)	male	tuberculosis	colocystoplasty	haematuria	signet-ring adenocarcinoma	colon
Stöckle <i>et al.</i> , 1986.	30 (60)	female	tuberculosis	ileocystoplasty	incidental finding of a mass	squamous cell carcinoma	bladder
Kawamura <i>et al.</i> , 1987.	14 (64)	male	not stated	ileocystoplasty	haematuria and irritative symptoms	adenocarcinoma	ileum
Stone <i>et al.</i> , 1987. Barrington <i>et al.</i> , 1997a.	5 (54)	female	tuberculosis	ileocystoplasty	pyelonephritis and impaired renal function	transitional cell carcinoma and carcinoma <i>in situ</i>	bladder near ileovesical anastomosis
Stone <i>et al.</i> , 1987. Barrington <i>et al.</i> , 1997a.	22 (53)	male	tuberculosis	ileocystoplasty	haematuria	adenocarcinoma	bladder near ileovesical anastomosis
Stone <i>et al.</i> , 1987. Barrington <i>et al.</i> , 1997a.	24 (42)	male	tuberculosis	ileocystoplasty	haematuria	adenocarcinoma	bladder near ileovesical anastomosis

Reference	Yrs from augmentation (pt. age at tumour)	Sex	Indication	Operation	Presentation of tumour	Pathology	Site of tumour origin
Hasegawa <i>et al.</i> , 1988. Hasegawa <i>et al.</i> , 1989.	29 (43)	female	tuberculosis	ileocystoplasty	renal failure secondary to ureteric stricture	adenocarcinoma	bladder multifocal
Golomb <i>et al.</i> , 1989.	24 (69)	male	chronic cystitis due to urethral stricture	sigmoid colocolocystoplasty	haematuria	transitional cell carcinoma	bladder and bowel
Golomb <i>et al.</i> , 1989. Golomb <i>et al.</i> , 1992.	8 (40)	female	hypospadias incontinence	ileocystoplasty	irritative symptoms	small cell carcinoma	bladder
Llarena Ibarguren <i>et al.</i> , 1989.	19 (56)	female	tuberculosis	colocystoplasty	UTI and haematuria	adenocarcinoma	bowel
Kadow <i>et al.</i> , 1989.	15 (53)	male	chronic granulomatous cystitis	caecocystoplasty	suprapubic pain and haematuria	adenocarcinoma	colovesical anastomosis
Kaneko <i>et al.</i> , 1989.	33 (57)	male	not stated	ileocystoplasty	haematuria	adenocarcinoma	ileum
Gregoire <i>et al.</i> , 1993.	8 (72)	male	hypocontractile bladder and urethral stricture	caecocystoplasty	haematuria	adenocarcinoma, transitional cell carcinoma and carcinoma <i>in situ</i>	bladder near caecovesical anastomosis
Lander and Pretorius, 1993.	5 (79)	male	transitional cell carcinoma	ileocaecocystoplasty	abdominal pain	adenocarcinoma	caecum and ileocaecal anastomosis
Takahashi <i>et al.</i> , 1993.	22 (67)	female	radiotherapy	ileocystoplasty	haematuria	adenocarcinoma	ileum
Tellez Martinez-Fornes <i>et al.</i> , 1993.	14 (66)	male	tuberculosis	caecocystoplasty	anorexia and loss of weight	adenocarcinoma	bowel ureteral anastomosis
Terao <i>et al.</i> , 1994.	25 (65)	female	not stated	ileocystoplasty	haematuria	adenocarcinoma	not stated
Shokeir <i>et al.</i> , 1995.	19 (42)	female	tuberculosis	colocystoplasty	haematuria and clot retention	transitional cell carcinoma	colovesical anastomosis
Fernandez-Arjona <i>et al.</i> , 1996.	25 (69)	female	tuberculosis	ileocystoplasty	haematuria and lumbar pain	mostly signet ring adenocarcinoma some squamous	bladder at ileovesical anastomosis involving ureter

Reference	Yrs from augmentation (pt. age at tumour)	Sex	Indication	Operation	Presentation of tumour	Pathology	Site of tumour origin
Jung <i>et al.</i> 1996.	36 (62)	male	tuberculosis	ileocystoplasty	haematuria	adenocarcinoma	ileum
Khawaja <i>et al.</i> 1996.	9 (24)	female	cyclophosphamide cystitis	caecocystoplasty	haematuria	adenocarcinoma	bladder
Barrington <i>et al.</i> 1997a	11 (33)	male	spina bifida	ileocystoplasty	haematuria	adenocarcinoma	bladder, near ileovesical anastomosis
Carr and Herschorn. 1997.	4 (26)	female	spina bifida	ileocystoplasty	bilateral hydronephrosis	signet ring adenocarcinoma	ileovesical anastomosis
Ishida and Koizumi. 1997.	38 (48)	female	tuberculosis	ileocystoplasty	recurrent pyelonephritis	adenocarcinoma	ileum, near ileovesical anastomosis
Koizumi <i>et al.</i> 1997.	37 (54)	female	tuberculosis	ileocystoplasty	not stated	adenocarcinoma	ileum, near ileovesical anastomosis
Louis <i>et al.</i> 1997.	36 (74)	male	tuberculosis	ileocystoplasty	haematuria	adenocarcinoma	ileum, near ileovesical anastomosis
Monnerat <i>et al.</i> 1997.	24 (34)	male	tuberculosis	ileocystoplasty	irritative symptoms	adenocarcinoma	ileum
Yoshida <i>et al.</i> 1998.	19 (53)	male	tuberculosis	ileocystoplasty	suprapubic pain	adenocarcinoma	ileum, near ileovesical anastomosis
Docimo <i>et al.</i> 1999.	20 (43)	male	posterior urethral valves	caecocystoplasty	haematuria	adenocarcinoma	colon, near colovesical anastomosis
El Otamy <i>et al.</i> 1999.	31 (60)	male	tuberculosis	ileocystoplasty	haematuria	squamous cell	ileum
Nahas <i>et al.</i> 1999.	25 (45)	male	tuberculosis	ileocaecocystoplasty	necropsy	adenocarcinoma	ileovesical anastomosis
Lane and Shah. 2000.	2 (74)	male	detrusor instability	ileocystoplasty	haematuria	transitional cell carcinoma	bladder
Lane and Shah. 2000.	3 (75)	male	detrusor instability	ileocystoplasty	haematuria	squamous cell	bladder
Sato <i>et al.</i> 2000.	40 (67)	female	tuberculosis	ileocystoplasty	haematuria	adenocarcinoma and transitional cell carcinoma	adenocarcinoma of ileum and transitional cell carcinoma of bladder
Ulmer <i>et al.</i> 2000.	40 (61)	male	tuberculosis	ileocystoplasty	renal failure and urgency	adenocarcinoma	bladder near ileovesical anastomosis

Reference	Yrs from augmentation (pt. age at tumour)	Sex	Indication	Operation	Presentation of tumour	Pathology	Site of tumour origin
Bono Ariño <i>et al.</i> 2001.	27 (49)	male	tuberculosis	colocystoplasty	irritative symptoms	adenocarcinoma	bladder near ileovesical anastomosis
Ali-El-Dein <i>et al.</i> 2002.	24 (43)	female	tuberculosis	ileocystoplasty	haematuria	transitional cell carcinoma	ileovesical anastomosis
Ali-El-Dein <i>et al.</i> 2002.	31 (58)	male	schistosomiasis	ileocystoplasty	haematuria and bladder mass	adenocarcinoma	ileovesical anastomosis
Ali-El-Dein <i>et al.</i> 2002.	13 (53)	male	schistosomiasis	ileocystoplasty	haematuria and loin pain	adenocarcinoma	lateral bladder adjacent to anastomosis
Moudouni <i>et al.</i> 2003	37 (68)	not stated	tuberculosis	ileocystoplasty	haematuria	squamous cell	ileum
Qiu <i>et al.</i> 2003	14 (73)	female	neuropathic bladder	gastrocystoplasty	haematuria	transitional cell carcinoma	stomach adjacent to anastomosis
Yokoyama <i>et al.</i> 2003	33 (65)	female	tuberculosis	ileocystoplasty	haematuria	adenocarcinoma	ileoileal anastomosis
Robles <i>et al.</i> 2004	16 (54)	female	interstitial cystitis	ileocystoplasty	weight loss and haematuria	adenocarcinoma	bladder near ileovesical anastomosis
Bruyneel <i>et al.</i> 2004	22 (59)	male	tuberculosis	ileocystoplasty	renal failure	small cell carcinoma	ileum
Soergel <i>et al.</i> 2004	21 (29)	not stated	myelomeningocele	ileocaecocystoplasty	haematuria	transitional cell carcinoma	bladder
Soergel <i>et al.</i> 2004	17 (37)	not stated	myelomeningocele	ileocaecocystoplasty	haematuria	transitional cell carcinoma	bladder near ileovesical anastomosis
Soergel <i>et al.</i> 2004	20 (44)	not stated	myelomeningocele	caecocystoplasty	bladder neck contracture	transitional cell carcinoma	uncertain
Baydar <i>et al.</i> 2005	14 (36)	male	neuropathic bladder	gastrocystoplasty	renal failure	signet ring adenocarcinoma	gastrovesical anastomosis
Nakata <i>et al.</i> 2005	17 (60)	female	tuberculosis	ileocystoplasty	pyrexial and unwell	transitional cell carcinoma	ileum
Ivil <i>et al.</i> 2006	8 (53)	female	spina bifida occulta	ileocystoplasty	haematuria	squamous cell	bladder
Berberian <i>et al.</i> 2006	20 (67)	male	radical cysto - prostatectomy	ileocystoplasty	dysuria and recurrent UTI	adenocarcinoma	ileum
Balachandra <i>et al.</i> 2007	10 (64)	male	spina bifida	gastrocystoplasty	haematuria	adenocarcinoma	stomach

Table 1.2. Benign tumours arising in bladder augmentations.

Reference	Yrs from augmentation (pt. age at tumour)	Sex	Indication	Operation	Presentation of tumour	Pathology	Site of tumour origin
Gepi-Attee <i>et al.</i> , 1992.	26 (53)	male	tuberculosis	sigmoid colocystoplasty	urinary frequency	villous adenoma	colon near colovesical junction
King <i>et al.</i> , 1992.	30 (50)	male	tuberculosis	ileocystoplasty	dysuria	tubulovillous adenoma	ileum
Goldman <i>et al.</i> , 1996.	5 (16)	male	lipomeningocoele	ileocystoplasty	incontinence and recurrent UTI	nephrogenic adenoma	multifocal tumour involving bladder and ileovesical junction
Redondo Martinez and Rey Lopez, 1998.	not stated (67)	male	transitional cell carcinoma	colocystoplasty	stricture at urethral anastomosis	nephrogenic adenoma	urethrocolonic junction
Yip <i>et al.</i> , 1999.	14 (38)	female	tuberculosis	caecocystoplasty	haematuria	villous adenoma	lateral wall of augmented bladder
Yamada <i>et al.</i> , 2006	44 (62)	female	tuberculosis	ileocystoplasty	haematuria	tubulovillous adenoma	multifocal tumour at the ileovesical junction

Adenocarcinoma is the predominant tumour type occurring following augmentation cystoplasty and has been reported in forty-one patients. In five patients the tumour was the signet-ring variant. In twenty-six of the forty-one tumours reported following ileocystoplasty the histology was an adenocarcinoma. Fourteen cases of transitional cell carcinoma have also been reported following augmentation cystoplasty. In three of these patients elements of transitional cell carcinoma were reported to coexist with the presence of another larger tumour. Seven cases of squamous cell carcinoma have been described and one of these was found to coexist with an adenocarcinoma (Fernandez-Arjona *et al*, 1996). In one case of transitional carcinoma, squamous carcinoma elements were reported (Smith and Hardy, 1971). There were two cases of small cell carcinoma (Golomb *et al*, 1997, Bruyneel *et al*, 2004) and one of leiomyosarcoma (Egbert *et al*, 1980) (Table 1.3). In thirty-three of the sixty cases the tumour occurring within the cystoplasty was stated to be at or near a urothelium bowel anastomosis. Unfortunately, in some cases the tumour location was not documented precisely and it is therefore not possible to be certain of the site of origin. Haematuria was the presenting symptom in thirty-nine of the sixty cases of carcinoma. Other presentations included deteriorating renal function, loin pain, the finding of an incidental mass on examination, the onset of irritative bladder symptoms, abdominal pain, pyrexia and a loss of weight (Table 1.4).

The number of cases of adenocarcinoma in ileal augmentations is striking as ileal tumours are rare (Watt *et al*, 1986). Adenocarcinoma of the large bowel is common affecting as much as six percent of the population (Seidman *et al*, 1985). However, the incidence of adenocarcinoma is between forty and sixty times less common in the small bowel than in the large bowel (Watt *et al*, 1986). Furthermore, adenocarcinoma of the urinary bladder is rare accounting for only 0.5 to 2 percent of

Table 1.3. Histology of tumours arising in bladder augmentations.

Adenocarcinoma	68%
Transitional Cell Carcinoma	23%
Squamous Cell Carcinoma	12%
Small Cell Carcinoma	3%
Leiomyosarcoma	2%

(Note that numbers exceed a total of 100% as some bladder augmentations contained more than one tumour type).

Table 1.4. Symptoms on presentation of patients with tumours arising in bladder augmentations.

Haematuria	65%
Irritative Bladder Symptoms	12%
Pain	8%
Deteriorating Renal Function	7%
Urinary Tract Infection	7%
Incidental mass	3%
Weight loss	3%
Pyrexia	2%
Urethral discharge	2%
Hydronephrosis	2%

(Note that numbers do not equal a total of 100% as some bladder augmentations presented with more than one symptom and in some case reports the method of presentation was not stated).

bladder neoplasms (El-Mekresh *et al*, 1998). Therefore, it is unusual to find such a high frequency of adenocarcinoma in augmented bladders using ileum. Carcinoma formation also occurs in experimental animals that have undergone enterocystoplasty. Adenocarcinomas have been observed in the presence of a urocolic anastomosis (Kalble *et al*, 1995; Crissey *et al*, 1980) and transitional cell carcinoma formation has been reported to occur in rats following augmentation cystoplasty (Spencer *et al*, 1993; Wu *et al*, 1995). Squamous carcinoma has been reported in urothelial patches transposed onto the sigmoid colon of rats (Aaronson *et al*, 1987).

The latency between an augmentation cystoplasty and the development of tumour is prolonged (mean: 20.4 years) but variable (range: two to forty years). Tumours occurring early after augmentation cystoplasty are of particular interest as many clinicians start screening for tumours ten years after surgery (Filmer and Spencer, 1990). Forty-four of the sixty patients developed a tumour fifteen years or more following operation. However, nine patients (fifteen percent) developed tumours less than ten years after their operation and these reports have particular implications on the design of screening programs. Lane and Shah (1999) have reported the earliest cases of tumour formation within an enterocystoplasty. They described a case of transitional cell carcinoma and one case of squamous cell carcinoma presenting two and three years respectively after ileocystoplasty formation. In both cases the tumours occurred within the native bladder segment away from the enterovesical anastomosis. Carr and Herschorn have provided the earliest description of an adenocarcinoma within an enterocystoplasty (Carr and Hershorn, 1997). These authors described a twenty-six year old woman with spina bifida and an ileal conduit since the age of seven years who underwent a hemi-Kock augmentation ileocystoplasty in which the existing ileal conduit was fashioned into the afferent

limb and the nipple valve whilst a fresh segment of ileum was used for the ileocystoplasty. Routine ultrasound four years after the operation demonstrated asymptomatic hydronephrosis and a poorly differentiated signet ring adenocarcinoma was found adjacent to the enterovesical anastomosis. It is of interest that the tumour arose close to the bowel harvested for this procedure, which is distant from the bowel that had been exposed for nineteen years to urine in the ileal conduit prior to augmentation.

The clam enterocystoplasty was described in 1982 for the treatment of lifelong day and night enuresis secondary to detrusor instability (Bramble, 1982) and is most commonly used for the management of refractory detrusor instability, primarily in middle-aged females (Mundy and Stephenson, 1985). However, the continuing development of a reliable artificial urinary sphincter has made reconstruction surgery effective in most motivated ambulant patients with neuropathic bladders and has resulted in younger patients undergoing the procedure (Stephenson and Mundy, 1985). As a result of the above developments in the last fifteen years, enterocystoplasty has been increasingly used for the management of an overactive bladder and in a younger population of patients (Barrington, 1998; López Pereira *et al*, 2006).

Tuberculosis was the stated reason for augmentation cystoplasty in thirty-two of the sixty cases of tumours arising within an enterocystoplasty reported in the literature. This predominance probably reflects the fact that tuberculosis was for many years the main use of the procedure (Dounis and Gow, 1979). However, it has been suggested that since the preponderance of tumours arising within an enterocystoplasty occur in patients who have undergone treatment for chronic

contracted tuberculous bladders, tuberculosis per se may be somehow implicated in the tumourigenic process and that inflammation caused by tuberculous organisms may either initiate or promote tumour growth (Dewolf, 1997).

Tuberculous cystitis is a recognised risk factor for carcinoma formation. Cancers occurring in the unoperated bladders of patients who have had tuberculous cystitis are almost exclusively squamous cell carcinomas (Muhlberger, 1991). However, of the thirty-two tumours described in patients where an enterocystoplasty was performed for the treatment of a tuberculous bladder, only five were squamous cell carcinomas. Two other patients developed carcinoma following augmentation cystoplasty for the treatment of a schistosomal contracted bladder (Ali-El-Dein *et al*, 2002). Squamous carcinoma is also a recognised complication of schistosomiasis, but both patients were found to have an adenocarcinoma. Tumours developing within an enterocystoplasty following tuberculous cystitis are commonly adenocarcinomas and are therefore unlikely to be initiated in the same way as classical squamous cell cancers, possibly because other factors which are inherent to the enterocystoplasty are more important in initiating tumourigenesis.

If tuberculosis was the major factor in the development of a tumour within an enterocystoplasty, then the increasing use of augmentation cystoplasty for the treatment of the overactive bladder coupled with the decreasing incidence of tuberculosis in western civilisation should mean that tumours are reported less often. However, the incidence of tumour formation continues to rise and is not entirely due to a long latent period between operation and tumour formation in tuberculous bladders. The proportion of reports of tumour formation within enterocystoplasties performed for the treatment of a tuberculous bladder is falling. In 1990 a review

identified fourteen reports of tumour formation within an augmentation cystoplasty. In ten cases (seventy-one percent) the operation was performed for treatment of a tuberculous bladder. In the last ten years there have been thirty-one reports of tumour formation within an enterocystoplasty and in fifteen cases (forty-eight percent) the operation was performed for a tuberculous bladder. Thus, about half of the recent reports of tumour formation within an enterocystoplasty have occurred in patients where the operation was performed for indications other than tuberculosis (Table 1.1). Patients developing tumours less than ten years after undergoing augmentation cystoplasty are likely to have bladders that are more susceptible to tumourigenesis than patients with a longer latent period between the time of operation and tumour formation. It is of interest that of the patients developing tumours less than ten years from the time of operation, only one of the nine patients underwent the procedure following genitourinary tuberculosis. Tumour formation occurred in eight of the nine patients in these patients in the absence of tuberculous inflammation. The inflammatory effects of tuberculosis may be contributory in those patients who have received an augmentation cystoplasty for the treatment of a contracted tuberculous bladder. However, factors other than tuberculosis initiate and promote tumourigenesis within enterocystoplasties and therefore carcinoma formation within enterocystoplasty will continue to be a problem in patients in whom the procedure is performed for other indications. There is often a long latent period between the time of operation and the presentation of clam tumours and it is expected that an increasing number of cases of tumour formation will be reported within enterocystoplasties originally performed to treat an overactive bladder.

Similarities have been found between tumours occurring in augmentation cystoplasty and those that occur at the anastamotic site following a

ureterosigmoidostomy (Husmann and Spence, 1990; Treiger and Marshall, 1991). Tumour formation at the ureterocolic anastomosis was first reported in 1929 (Hammer, 1929). Although ureterosigmoidostomy has been commonly used since 1920, the increased risk of adenocarcinoma of the sigmoid colon following the procedure was not fully appreciated until the late 1970s (Leadbetter *et al*, 1979). It has been noted that in the first forty years in which bladder augmentation was in general use, fourteen tumours were noted (Couvelaire, 1950, Filmer and Spencer, 1990). Similarly, fourteen ureterosigmoidostomy carcinomas were detected by 1966, i.e. forty-six years after this form of diversion came into general use (Urdaneta *et al*, 1966). Sixty carcinomas in augmentation cystoplasties are now recorded in the literature, fifty-seven years since the operation has been in general use. Fifty-nine years after ureterosigmoidostomy was in general use forty-five cases of carcinoma had been detected (Leadbetter *et al*, 1979). The increase in the reported cases of tumour formation following an enterocystoplasty parallels that observed after the introduction of ureterosigmoidostomy. The similarity is both striking and alarming. In the absence of a formal registry or prospective studies on defined populations, the incidence of tumours in patients with ureterosigmoidostomy was unknown for many years (Gittes, 1986). However, although tumour formation in a ureterosigmoidostomy was first described in 1929 the significance of this finding was not appreciated until as late as 1975 (Hohenfeller, 1992). It is important that the true incidence of carcinoma in augmentation cystoplasty is recognised in order to make informed decisions on the management of patients with an overactive bladder and patients who have already undergone an augmentation cystoplasty.

The incidence of carcinoma after ureterosigmoidostomy has been estimated at two to fifteen percent (Azmuddin *et al*, 1999) with the incidence of polyps (forty

percent) being much higher (Stewart, 1986). Several authors have quoted the average interval from the time of ureterosigmoidostomy to the development of tumours as being between twenty to twenty-six years with a range of two to fifty-three years (Azmuddin *et al*, 1999). The majority of tumours (sixty-nine percent) were diagnosed fifteen to thirty years after the ureterosigmoidostomy (Sheldon *et al*, 1983). The increased risk of tumour in ureterosigmoidostomy has been variously quoted to be increased 100 fold (Stewart, 1986), 500 fold (Iannoni *et al*, 1986) and 7,000 fold (Gittes, 1986). If there is a similar malignant transformation following the clam procedure, this would equate to a carcinoma induction rate of up to 29% (Kalble, 1993). However, the individual risk of developing adenocarcinoma in ureterosigmoidostomy varies with the age of the patient when the surgery was performed. Thus, ureterosigmoidostomy performed in younger patients (twenty-five to thirty years) increases the risk of adenocarcinoma formation by a factor of 265, while the risk of adenocarcinoma is only increased by eight fold when surgery is performed between the ages of fifty-five and sixty (Kalble, 1996). Generally, ureterosigmoidostomy will be *in-situ* for a longer period of time in younger patients who will consequently be exposed to potential carcinogens throughout their life resulting in an increased risk of carcinogenesis compared to older patients.

In one series, the incidence of tumour formation was found to be at least 1.5 percent in sixty-four patients who had undergone augmentation cystoplasty and followed up for 15 years or longer (Filmer and Spencer, 1990). In a second series, 645 patients with a urinary intestinal anastomosis were followed for a minimum of four years (Ali-El-Dein *et al*, 2002). One percent of these patients developed a late malignancy in the bowel segment. However, the incidence of cancers developing in ileocystoplasties (5.5 percent) was much higher than tumour formation in patients

with an ileal loop conduit (0.3 percent) or an ileal replacement of ureter (0.8 percent). In this study three out of fifty-five patients who had undergone an ileocystoplasty developed cancers, all situated adjacent to the enterovesical anastomosis. However, it seems likely that when patients are followed-up over a longer period of time a higher incidence of tumour formation will be found. In a third series, 260 paediatric patients who had undergone treatment for a neuropathic bladder were followed-up at least ten years after undergoing an augmentation enterocystoplasty (Soergel *et al*, 2004). The incidence of carcinoma formation within an enterocystoplasty was found to be at least 1.2 percent. However, if the latent period between operation and carcinoma formation was assumed to be twenty to twenty-five years then the relative risk of cancer formation in this study was 3.8 percent.

Therefore, at present the precise incidence of tumour transformation within augmentation cystoplasty is unknown. The number of procedures that have been performed is unknown, and hence, it is only possible to estimate the likely incidence of tumour formation in augmentation cystoplasty. Furthermore, it is unknown whether the risk of malignancy following enterocystoplasty will vary in the changing patient population undergoing this procedure. Nevertheless, the previous experience with urocolic tumours must serve as warning that there is a possibility of more tumours developing over the next ten to twenty years in patients who have undergone an enterocystoplasty.

The histology, localisation and the time period between operation and development of tumours in augmentation cystoplasty are similar to those following ureterosigmoidostomy. Despite the similarities in the pathology and natural history of tumours occurring following augmentation cystoplasty and ureterosigmoidostomy

there are also differences between the types of cancer. Tumours within an augmentation cystoplasty, unlike those arising in an ureterosigmoidostomy may be found distant from the anastomosis and are sometimes multifocal. Furthermore, benign adenomatous polyps are a common finding following an ureterosigmoidostomy (Stewart, 1986) but are rarely reported in an augmented bladder (Table 1.2). In addition, ureterosigmoidostomy tumours are almost entirely adenocarcinomas whereas cystoplasty tumours display a more variable histology. The reasons for the differences between tumours following augmentation cystoplasty and ureterosigmoidostomy cannot be readily explained.

Carcinoma formation within an enterocystoplasty has a low incidence and a long latent period between operation and cancer formation creating immense difficulties when defining the aetiology, natural history and epidemiology of the disease. Previous publications have highlighted the need for a database to monitor the incidence of tumour formation in an augmentation cystoplasty (Filmer and Spencer, 1990; Shaw and Lewis, 1999) since such tumours occur in a sporadic manner and there is no reliable method of reporting their occurrence. Anecdotally, it is understandable that the majority of journals do not consider publishing isolated reports any longer since augmentation tumours are well described although occasionally, case reports do appear in lesser-known journals. However, counting case reports provides only a crude measure of the numbers of tumours that have occurred in augmentation cystoplasties and the true incidence of malignant tumours in augmented bladders using bowel is unknown.

1.3.2. Tumour surveillance.

Many clinicians perform lifelong follow-up on patients who have undergone lower urinary tract reconstruction surgery, primarily to monitor kidney function. Many clinicians also screen their patients for the development of malignancy in the reconstructed bladder. However, currently there is no consensus of what a screening program should consist of or when screening should be started for an individual. Some surgeons recommend screening at annual intervals from ten years after surgery (Filmer and Spencer, 1990), others start screening at five years (Kalble, 1993) but many do not perform any screening for malignancy (Shaw and Lewis, 1999). Similarly, there is controversy concerning when to start colonoscopic screening in patients following the construction of a ureterosigmoidostomy. Some clinicians recommend screening from five to six years after operation (Bissada *et al*, 1995), others from twelve years (Koo *et al*, 1996), and others suggest that surveillance colonoscopy should be started soon after ureterosigmoidostomy because the onset of cancer polyps can be as early as two years (Starling *et al*, 1984). How often patients should be screened for possible tumour formation following ureterosigmoidostomy is also controversial. Some clinicians advocate annual screening (Bissada *et al*, 1995) whilst others recommend biennial screening (Koo *et al*, 1996). It is difficult from the available data to ascertain the optimal frequency of screening for tumours arising in augmentation cystoplasties, but it is worthy of note that the literature records only three tumours that have been detected in patients who were in a screening program (Ali-El-Dein, 2002). Furthermore, even in these three patients the tumours did not present incidentally on surveillance screening, but were diagnosed after further investigations had been initiated following presentations with haematuria. Therefore, currently although there is a theoretical basis for screening patients for tumours following augmentation cystoplasty and a general clinical consensus that screening

patients is prudent, there is as yet no evidence that screening programs are effective in the early detection of these often-aggressive tumours.

Endoscopy with or without biopsy is likely to be the most sensitive method of surveillance. The presence of polyps or other evidence of premalignant change would allow for the identification of patients at an increased risk of tumourigenesis. However, the natural history of these tumours is not yet known and it has not been established whether premalignant changes predispose to the earlier detection of a tumour. In our unit biopsies are taken from close to the anastomosis for histopathological investigation, but as yet, this has so far proved unhelpful for the early diagnosis of tumours.

Urinary cytology is a non-invasive procedure and may be helpful in diagnosing malignant change. A high sensitivity and specificity can be achieved by an experienced cytopathologist but the general consensus of opinion is that urinary cytology is not capable of detecting all cases of recurrent malignant disease (Ross and Cohen, 2001). Infection and inflammation are commonly found in the bladders of patients who have undergone an enterocystoplasty and can make urinary cytology specimens difficult to interpret because such conditions increase the number of false positives and false negatives.

Flow cytometry has been investigated as means of detecting urothelial tumours. Flow cytometry requires a large number of well-preserved cells and specimens are usually obtained by washing the urinary bladder. This technique is particularly useful in the screening of high-risk populations and for the monitoring of patients with a known history of urothelial carcinoma of the bladder (Wheeless *et al*,

1993). However the use of flow cytometry for the detection of tumours is limited since only significant DNA chromosomal abnormalities such as an addition or deletion of the equivalent of two large chromosomes can lead to changes in DNA ploidy detectable by this technique. Although essentially an experimental technique at present, several studies have reported that changes in DNA ploidy detected by flow cytometry correlate well with cystoscopic and cytology findings (Klein *et al*, 1982, Giella *et al*, 1992). Furthermore, continued use of flow cytometry and urine cytology together improves the accuracy of diagnosis by increasing sensitivity (Murphy *et al*, 1986).

Docimo *et al* (1999) described loss of heterozygosity using microsatellite analysis of tumour cells in the urine of a patient obtained before surgical resection of an adenocarcinoma occurring in an augmentation cystoplasty. This observation suggests that microsatellite urine analysis may be useful for the detection of tumours arising in augmentation cystoplasties. Microsatellites are inherited short tandem repeat DNA sequences with low mutation rates unique to individuals (Brentnall, 1995). Assays for abnormalities in microsatellites generally include the detection of mutational copy errors and deletions of gene loci seen as loss of heterozygosity. Studies of microsatellite instability have demonstrated a sensitivity of eighty-three to ninety-five percent and a specificity of one hundred percent for the early detection of recurrence of urothelial neoplasia in bladder washings and urinary cytology specimens (Ross and Cohen, 2001). Microsatellite analysis on stored frozen urine specimens is reported to be twice as sensitive as urine cytology (Steiner *et al*, 1997). Therefore, microsatellite analysis may be useful as an adjunct to the early detection of urothelial cancer and may prove useful in the screening of enterocystoplasty tumours but this suggestion warrants further investigation.

A variety of other immunoassays, cytogenetic and molecular techniques have been designed to complement cytology and improve the overall sensitivity and specificity for the detection of urothelial cancer (Ross and Cohen, 2001). Therefore, although at present no new individual marker has been shown to eliminate the need for follow-up cystoscopy, there is a consensus that cytogenetic and molecular techniques increase the sensitivity and specificity of cytology and may increase the intervals between surveillance cystoscopy for the management of urothelial neoplasia (Wiener *et al*, 1998). However, enterocystoplasty tumours appear to be different in character to most urothelial neoplasms and the natural history and mechanisms of tumourigenesis are not fully understood. Since these tumours are generally aggressive it may be inappropriate to increase the length of time between surveillance cystoscopy for a patient with an enterocystoplasty. Clearly, further studies are required to determine the optimal method of screening for tumours in patients who have undergone augmentation cystoplasty.

1.3.3. Pathogenesis.

The precise mechanisms involved in the pathogenesis of tumour formation associated with enterocystoplasty are not known. There is however much evidence to suggest that an augmentation cystoplasty may potentiate malignant transformation and tumour growth.

Tumour formation is commonly described at the junctional zone of the enterovesical anastomosis and histological changes have been described in this area. Rats killed at least one year after augmentation cystoplasty were shown to display hyperplastic and metaplastic changes at the junctional zone in the tissue located at

the area of the enterovesical anastomosis where dissimilar tissues had been opposed (Buson *et al*, 1993; Little *et al*, 1994). The observed metaplastic changes were characterised by pronounced glandular metaplasia and cystic dilatation. Transitional cell carcinomas have also been reported at the junctional zone in rats following an ileocystoplasty or colcystoplasty (Spencer *et al*, 1993).

Tumours occurring after construction of an enterocystoplasty are often located close to the enterovesical anastomosis suggesting that the inflammatory response which occurs in this area may predispose to malignant transformation. It has been previously suggested that chronic inflammation per se enhances nitrosation secondary to activation of macrophages and neutrophils (Dull *et al*, 1988). Promoters are substances that augment tumourigenesis that has been induced by primary carcinogens such as N-nitroso compounds. These promoter substances need not be mutagenic but may act in an epigenetic manner, for example by increasing the risk of cumulative mutations by sustaining cell proliferation (Ames and Gold, 1990). Activated macrophages have been shown to promote malignant transformation *in vitro* via the production of active oxygen species (Weitzman *et al*, 1985). Other studies have shown that macrophages produce nitrate and nitrite and catalyse nitrosamine formation in the presence of a secondary amine when they are stimulated by *Escherichia coli* (*E. coli*) lipopolysaccharide or interferon (Stuehr and Marletta, 1987), a process that does not require the production of reactive oxygen species. Some factors such as nitrosamine, sutures (Chester *et al*, 1987), stones (Chapman *et al*, 1973) and urinary epidermal growth factor (Yura *et al*, 1989) have been shown to act as tumour promoters by enhancing the effect of an initiator. If a cystoplasty is inflamed as is frequently seen on cystoscopy (Nurse and Mundy, 1989b),

inflammation may be a significant factor in the aetiology of tumourigenesis in these patients.

The junctional zone between the bowel and bladder mucosa has been identified as a possible site for tumour initiation and consideration has been given to aberrant stromal-epithelial signalling as a mechanism of tumourigenesis. Mesenchymal-epithelial interactions are a requirement for the development of bladder smooth muscle (Baskin *et al*, 1996). Furthermore, there is experimental evidence suggesting that these interactions are not limited to the embryo but do occur later in life. Thus, it has been shown that both older urothelium and heterotypic epithelium can induce smooth muscle differentiation in bladder mesenchyme (DiSandro *et al*, 1998). In an augmentation cystoplasty, the mesenchyme and epithelium of the bowel and bladder are placed in direct apposition. Consequently, as reciprocal cell to cell interactions occur between the epithelium and mesenchymal tissues, the cells of these tissues may be affected by aberrant cell signalling which may affect the regulatory mechanisms of epithelial cell growth and predispose to malignancy. It is possible that chronic disturbances in cell signalling and function in the region of the enterovesical anastomosis may explain the development of epithelial metaplasia and tumours in this area of the bladder. The hypothesis that perturbed epithelial-stromal cell-cell interactions can result in tumour formation following augmentation cystoplasty is not unique, similar mechanisms having been suggested to be involved in the development of both colon and prostate carcinomas (Zweibaum *et al*, 1984; Cunha *et al*, 1996).

The tissue of origin for tumour formation in a clam enterocystoplasty is unclear. It has been suggested that because of the variable histological types of

clinical and experimental tumours, the epithelium of origin may vary between individuals (Spencer and Filmer, 1993). There is difficulty in obtaining specimens of these rare tumours and therefore there are only a few series where the tissue of origin of such cancers has been examined. In a series of four tumours from our own unit the tumours were found to be primarily on the bladder side of the anastomosis (Barrington *et al*, 1997a). Histological examination revealed that the urothelium displayed glandular metaplasia and dysplasia with intestinalization overlying normal detrusor muscle, observations suggesting that the urothelium is the site of origin for tumours occurring after enterocystoplasty. This hypothesis is supported by immunohistochemical studies of the epithelium of the bladder, bowel and enterovesical anastomosis in dogs with an ileocystoplasty which indicated overgrowth of hyperplastic transitional cell epithelium at the enterovesical junction, the cells expressing both uroplakins and mucosubstances, observations which suggest that migrated hyperplastic epithelial cells undergo changes characteristic of ileal epithelium (Gitlin *et al*, 1999). In contrast, immunohistochemical assessment of five enterocystoplasty tumours demonstrated that the bladder mucosa distant from the tumour did not express any significant mucin secretion whereas cystoplasty bowel distant from the tumour displayed features characteristic of the parent orthotopic bowel (Murray *et al*, 1995). Since the tumours arising in ileocystoplasties exhibited a predominant sulphomucin characteristic of bowel mucosa, the authors suggested that the tumours were of intestinal origin (Murray *et al*, 1995) but pointed out benign conditions such as cystitis cystica (Newbould and McWilliam, 1990) and transitional cell carcinoma (Barresi and Marafioti, 1990) can also exhibit this pattern of staining.

The changes in mucosal morphology which occur in an enterocystoplasty after reservoir construction may be important for the long-term stability of the mucosa. Since intestinal mucosal cells metabolise both circulating and luminal metabolic substrates, changes in luminal metabolic substrate availability may disrupt the metabolic pathways of otherwise healthy mucosa. For example, the metabolic flux of butyrate is reduced in ileocystoplasty bowel mucosa compared to normal bowel mucosa (Duffy *et al*, 1998) reducing butyrate metabolism and resulting in downregulation of the enzymatic pathways involved in butyrate metabolism. This reduction in butyrate metabolism may have important implications for the morphological stability of the reservoir mucosa since butyrate is known to stabilise enteric mucosa (Whitehead *et al*, 1986). Metabolic acidosis commonly occurs after enterocystoplasty (Boyd *et al*, 1989) and increases the exposure of enteric mucosa to ammonia which induces morphological changes in the epithelium (Vissek, 1978). Consequently, the loss of the cytoprotective effect of butyrate (Tanaka *et al*, 1989) may render the mucosa more susceptible to proliferation and neoplastic change.

Experimental evidence on the mechanisms of carcinogenesis has shown that the process occurs in several stages (Knudson, 1986). It seems likely that changes occurring at the junctional zone produce an epithelium that is susceptible to endogenous or exogenous carcinogens which act as carcinogenic initiators causing irreversible changes in DNA. Other substances may act as promoters by enhancing the effects of initiators. The exact mechanisms involved in tumourigenesis in an enterocystoplasty in man may be difficult to identify due to the long latent interval between exposure to carcinogens and tumour formation. Patients with a clam cystoplasty may well be more at risk from carcinoma formation than patients undergoing substitution cystoplasty since the junctional zone in the region of the

urothelial bowel anastomosis is greater with the former procedure. It has been hypothesised that contact between urine, faeces, urothelium, colonic epithelium and a healing anastamotic site activates faecal carcinogens, thus initiating the process of carcinogenesis in ureterosigmoidostomy (Gittes, 1986). In particular, Gittes suggested that the presence of chronic bacteriuria and nitrosamines might be responsible for carcinogenesis in patients who undergo ureterosigmoidostomy. However, subsequent work has shown that direct anastamotic contact with the faecal stream is not a prerequisite for tumour formation (Shands *et al*, 1989).

N-nitroso compounds are highly carcinogenic compounds that have been shown to induce carcinomas particularly in the stomach, bladder and augmentation cystoplasty. N-nitroso compounds may be divided into the N-nitrosamines and the N-nitrosamides. In general the nitrosamines require transformation by enzymes to active intermediates. N-Nitrosamines are potent carcinogens (Magee and Barnes, 1956, Peto *et al*, 1991) which are produced endogenously in the stomach and the bladder and occur widely in the environment. The nitrosamides are not stable at physiological pH and probably decompose to active intermediates analogous to those derived from nitrosamines. The acute toxicity of nitrosamines decreases with the chain length and therefore small chain compounds are thought to be more important in carcinogenesis. Metabolic activation of nitrosamines results in the formation of alkylation products that can combine with DNA to form abnormal adducts (Shank, 1975).

Promutagenic changes in the DNA of patients with schistosomiasis are associated with elevated urinary nitrosamine levels and an increased incidence of bladder cancer (Badawi *et al*, 1995). This promutagenic adduct (O⁶-methylguanine)

is specifically repaired by the enzyme O⁶-alkylguanine-DNA alkyltransferase (Badawi *et al*, 1994). Endoscopic biopsies of ileocystoplasties have shown that there are large amounts of this enzyme in the bowel segment but only small amounts in the transitional epithelium (Barrington *et al*, 1997a). This finding suggests that the bowel mucosa is protected against nitrosamine-induced alkylation damage and hence, the initiation of carcinogenesis and supports the hypothesis that tumours arise from the transitional epithelium rather than the bowel.

Nitrates are normal constituents of human urine and it is well established that certain bacteria, particularly gram-negative bacteria, can metabolise nitrate to nitrosamines (Hawksworth and Hill, 1971; Calmels *et al*, 1985). Urinary nitrosamine levels are significantly elevated in patients with urinary tract infections (Radomski *et al*, 1978; Calmels *et al*, 1985) and there is epidemiological evidence of a link between chronic urinary tract infection and the development of bladder cancer (Kantor *et al*, 1984). Patients who have undergone a clam enterocystoplasty have a high incidence of urinary tract infections (Fenn *et al*, 1992) and many patients are undergoing surgery at a younger age and have a longer life expectancy after their surgery. Therefore, the vesicoenteric anastomosis is likely to be exposed to several episodes of elevated nitrosamine levels which may increase the risk of carcinogenesis.

An elevation in urinary nitrosamine levels has been reported in patients with ileal reservoirs, rectal bladder, ileal and colonic conduits, ureterosigmoidostomies and enterocystoplasties (Tricker *et al*, 1989, Nurse and Mundy, 1989b, Kalble *et al*, 1990, Groschel *et al*, 1992). Nurse and Mundy (1989b) demonstrated that when urinary nitrosamine levels were particularly high following augmentation cystoplasty,

bacteriuria almost invariably existed along with chronic inflammatory changes in the cystoplasty and in some of these patients, histological premalignant changes were present. The duration of the cystoplasty does not appear to have any influence on urinary nitrosamine levels (Woodhams *et al*, 2001).

Creagh *et al* (1997) observed no differences in the nitrosamine content of urine of patients who had undergone augmentation cystoplasty and those of normal controls. However, if the cystoplasty was inflamed, and particularly if the inflammation was accompanied by infection, urinary nitrosamines were increased significantly. These results suggest that patients with an inflamed enterocystoplasty and an accompanying infection are most at risk from malignant change. This hypothesis assumes however, that nitrosamines are directly related to the development of tumours in an enterocystoplasty.

Antibiotics have been shown to reduce urinary nitrosamine levels in patients who have undergone enterocystoplasty (Greenwell *et al*, 2001b, Woodhams *et al*, 2001). Antibiotic treatment of patients with an enterocystoplasty who have infected urine also reduces nitrosamine levels (Greenwell *et al*, 2001b). Similarly, patients with an enterocystoplasty given antibiotic prophylaxis have also been shown to have significantly lower nitrosamine levels than untreated patients (Woodhams *et al*, 2001). Furthermore, those patients receiving prophylactic antibiotics had nitrosamine levels similar to those found in normal controls (Greenwell *et al*, 2001b). This observation suggests that if urinary nitrosamines are promoting malignancy in enterocystoplasties, prophylactic antibiotics may reduce this risk. A reduction in nitrosamine levels was particularly noted in the urines of patients treated with trimethoprim, ampicillin or cefadroxil compared to those receiving nitrofurantoin

and septrin (Greenwell *et al*, 2001b). Consequently, it has been suggested that patients with persistent urinary tract infection should be treated prophylactically with trimethoprim, ampicillin or cefadroxil. However, experience in our own unit has suggested that patients with an augmentation cystoplasty are prone to profound problems with antibiotic resistance if treated by prophylactic antibiotics. Therefore, in our unit antibiotic use in patients with an augmentation cystoplasty is limited to those patients who have symptomatic urinary tract infections proven by urinary culture.

The effects of N-butyl-N- (4 hydroxybutyl) nitrosamine (BBN), administered for twelve weeks via the drinking water, in rats with small bowel transplanted into their urinary bladders produced bladder tumours of various grades after thirty weeks (Wu *et al*, 1995). Two rats in which the bladder had been augmented with jejunum, developed transitional cell bladder carcinoma, one of which had invaded the adjacent jejunal mucosa. The remaining tumours were adenocarcinomas of the bowel mucosa. These findings suggest that the bladder is more susceptible to the effects of nitrosamines than the bowel, supporting the hypothesis that cancers in augmentation cystoplasty arise from bladder rather than bowel.

Incomplete bladder emptying, mucus production and altered composition of urine contribute to the increased bacterial colonisation of the urine following augmentation cystoplasty. Furthermore, it has recently been suggested that preferential adherence of bacteria to ileal rather than bladder mucosa after augmentation cystoplasty may be contributory to bacterial colonisation (Nakano *et al*, 1999). *E. coli* with type 1 pili and *E. coli* C5 were found to preferentially adhere to ileum rather than bladder in rats with an augmentation cystoplasty and inoculated

transurethrally with bacteria (Nakano *et al*, 1999). Type 1 fimbriated cells adhere to human buccal epithelial cells, intestinal cells and vaginal cells (Sauter *et al*, 1993) and their presence in the ileum may be responsible for the adherence of bacteria to the small bowel rather than bladder. Further work has suggested that a colonic segment offers more resistance to *E. coli* adherence than an ileal segment when used for augmentation cystoplasty in rats (Sakai *et al*, 2000) and may partly explain the lower incidence of reported tumours in augmentation using large bowel. However, these observations contrast with findings in man where bacteriuria and nitrosamine levels have been found to be higher in patients with caecocystoplasties compared to those with ileocystoplasties (Nurse and Mundy, 1989b). These findings may explain, at least in part, the persistent bacteriuria observed in patients with an ileocystoplasty. *E. coli*, the organism most commonly implicated in urinary tract infections (Fenn *et al*, 1992), can synthesise nitrosamines from secondary amines (Calmels *et al*, 1985). Furthermore, *E. coli* urinary tract infections in patients who have undergone enterocystoplasty are associated with higher nitrosamine levels than urinary tract infections caused by other organisms (Greenwell *et al*, 2001b). These observations suggest that *E. coli* infections of the urinary tract may, via the ability of these bacteria to promote the formation of nitrosamines, may be involved, at least in part in the development of carcinogenesis associated with an enterocystoplasty.

Gastrocystoplasty is used in some centres to augment the bladder, particularly in children (Dykes and Ransley, 1992). Recently, three cases of carcinoma formation within a gastrocystoplasty have been described. In the first case a transitional cell carcinoma was present at the enterovesical anastomosis (Qiu *et al*, 2003). In the second case a signet ring cell variant of adenocarcinoma was found at the anastomosis (Baydar *et al*, 2005). Finally, in the third case an adenocarcinoma was

present in the gastric remnant (Balachandra *et al*, 2007). Experimental studies in rats have shown that transitional metaplasia and papillary hyperplasia occur more frequently in a gastrocystoplasty compared to either ileocystoplasty or sigmoid colocolocystoplasty, although the malignant potential of these lesions is unknown (Buson *et al*, 1993). Furthermore, in rats the incidence of tumour formation in a gastrocystoplasty is greater than either an ileocystoplasty or a colocolocystoplasty (Little *et al*, 1994). Nitrosamines are produced endogenously by both the stomach and the bladder and have been implicated in both stomach and bladder cancer but not in colon cancer (Shank, 1975). These observations suggest that it may not be advisable to use gastric segments in augmentation cystoplasty.

Human basic fibroblast growth factor (BFGF) is present in a wide range of tissues and has a physiological role in wound healing. BFGF is angiogenic and its autocrine and paracrine proliferative effects are thought to be involved in the growth and progression of a wide range of cancers. Patients with bladder tumours have elevated circulatory levels of BFGF (Nguyen *et al*, 1993) and it has been suggested that the measurement of urinary levels of this growth factor may be useful in predicting cancer progression and monitoring the effects of chemotherapy (Nguyen *et al*, 1994). Urinary levels of BFGF are higher in patients who have undergone clam ileocystoplasty compared to normal controls (Barrington *et al*, 1996b). In patients with an augmented bladder, the incorporated bowel segment is often chronically inflamed at cystoscopy and biopsy (Nurse and Mundy, 1989b), suggesting that BFGF may be released from the extracellular matrix of the inflamed bowel segment of the cystoplasty. BFGF binds strongly to heparin which is a constituent of the glycosaminoglycan (GAG) layer on cell membranes. Synthetic GAGs such as pentosan polysulphate (PPS) have been shown to reduce the rate of tumour

implantation and growth (Zugmaier *et al*, 1992). In patients with a clam ileocystoplasty PPS markedly reduced symptoms of bladder pain and frequency and mucus production but had had no effect on urinary BFGF levels (Barrington *et al*, 1996a). However, BFGF was significantly reduced in patients who were symptomatically improved compared to those unresponsive to PPS (Barrington *et al*, 1996a). For the present time however, there is insufficient information to determine whether PPS can reduce the incidence of tumour formation in augmentation cystoplasty.

A deficiency in the trace element selenium is associated with an increased incidence of bladder cancer (Salonen *et al*, 1984; Lange, 1991). There is epidemiological evidence to suggest a higher incidence and increased mortality from cancer in geographical areas poor in selenium (Hocman, 1988). Furthermore, reduced plasma levels of selenium have been shown to increase susceptibility to cancer in rats treated with carcinogens such as nitrosamines (Clark, 1985). Selenium is essential for the activity of the enzyme glutathione peroxidase which reduces the deleterious effects of free radical damage in healthy individuals by converting hydrogen peroxide to water. It is thought that the hydroxyl radical is capable of producing single- and double-strand breaks in DNA which can lead to mutagenesis and carcinogenesis (Lunec, 1990). Serum selenium levels are reduced in patients with an ileocystoplasty compared to healthy controls (Barrington *et al*, 1997b). However, this difference is only found in younger patients less than fifty years of age. A similar reduction in plasma selenium levels has also been reported in patients with a neuropathic bladder who had not undergone ileocystoplasty (El-Masri and Fellows, 1981). This observation is of interest since it is known that neuropathic patients have a twenty fold increased risk of developing bladder cancer compared to normal

controls (Davies, 1977). In an augmentation cystoplasty, it is possible that superoxide radicals are generated by inflammatory phagocytes and that plasma selenium levels are reduced as a result of their increased use to neutralise these free radicals (Barrington, 1998). However, the precise effects of increased free-radical production on tumourigenesis in patients who have undergone an enterocystoplasty are uncertain. In summary, there is good evidence that enterocystoplasty predisposes to malignant transformation and tumour growth although the precise mechanisms involved are not clear.

1.4. The genetic basis of carcinogenesis in clam enterocystoplasty.

There is only limited information on the genetic changes occurring at the enterovesical anastomosis in patients who have undergone a clam ileocystoplasty. Comparative Genomic Hybridisation (CGH) is an *in situ* hybridisation technique that detects chromosomal amplifications and deletions in the entire genome (Kallioniemi *et al*, 1992). CGH offers many advantages over conventional cytogenetic analysis as it does not require cellular preparations. Until the advent of CGH most cytogenetic analysis was confined to haematological malignancies since these malignant cells have a high mitotic rate and are easily prepared as an *in-vitro* cell culture. CGH has been used to screen virtually all solid tumours for chromosomal changes and has revealed, in all instances, a tumour-specific blueprint of chromosomal copy number changes (Forozan *et al*, 1997). CGH is not dependent on the tumour source and it requires only DNA extracted from cell lines or from fresh, frozen or paraffin-embedded tissue. The applicability of CGH to archival material allows retrospective analysis of many tumours that have been pathologically well characterised and where the clinical outcome is known.

In the CGH assay, DNA is extracted from a test sample (usually tumour) and from normal tissue. These samples are differentially labelled with green (tumour DNA) and red (control DNA) fluorochromes and are competitively hybridised in equal amounts onto a normal metaphase. The hybridisation along the axis of each of the chromosomes is then analysed. The relative amount of tumour and reference DNA at a given locus are dependent on the relative abundance of those sequences in the two DNA samples. The fluorescence ratio of the two fluorochromes is quantified by computation to determine whether regional losses or gains have occurred (Weiss *et al*, 1999).

CGH is a method whereby information on the whole genome can be obtained using a small amount of DNA. The technique does however have some limitations since it cannot detect balanced translocations and does not provide any information of tissue architecture. The detection of amplifications and deletions by CGH is related to chromosome sites and due to the repetitive nature of centomeres and telomeres, copy numbers in these regions are poorly detected. The lowest resolution of an over-represented DNA sequence is 0.25 megabases (Mb); the maximum resolution is 1 Mb for loss of two homologues and 2 Mb for loss of one homologue (Zitzelsberger *et al*, 1997).

CGH is suitable to screen tumours for alterations in cellular DNA and has on occasions been used for the analysis of premalignant tissue (Willenbacher *et al*, 1997; Walch *et al*, 2000). A preliminary study using CGH on DNA extracted from clam anastomotic biopsies reported amplifications of DNA on the short-arm of chromosome 18 in three out of fifteen patients and on the long-arm of chromosome 9 in two out of fifteen patients (Appanna *et al*, 2000, Appanna, 2004). No amplifications were detected in control specimens from the native bladder remnant (Appanna *et al*, 2000, Appanna, 2004). It seems likely therefore that large areas of clonal change were present in the bladder biopsies studied since CGH requires that at least sixty percent of nuclei contain the same genetic alteration for an abnormality to be detected (Kallioniemi *et al*, 1994). It is possible that cellular or DNA changes were also present in those patients who did not display amplifications but this was not of a sufficient extent to be detected by CGH. If histological changes such as dysplasia are present then microdissection techniques can be used to increase the sensitivity of CGH (Schütze and Clement-Sengewald, 1994) although reliable results

have been obtained from premalignant tissue when this has not been possible (Willenbacher *et al*, 1997). If histological changes are present at the enterovesical anastomosis in patients who have undergone enterocystoplasty, then they are usually those of generalised inflammation and therefore, microdissection would not have been helpful in identifying localised areas suitable for analysis.

In patients with a clam ileocystoplasty, previous studies using CGH identified abnormalities of chromosomes 9 and 18 from tissue taken adjacent to the enterovesical anastomosis, observations which suggest that the urothelium is genetically unstable (Appanna *et al*, 2000, Appanna, 2004). These genetic alterations were present in patients in whom there was no evidence of carcinoma and longitudinal studies are required to confirm whether genetic abnormalities accumulate with time. Amplifications of chromosomes 8p and 21q have been reported in two clam cancers using CGH suggesting that instability of these chromosomes may also be important in the development of cancers within augmentation cystoplasties (Appanna *et al*, 2000, Appanna, 2004).

Although CGH is a useful cytogenetic technique that assesses chromosomal alterations on a global scale, its sensitivity is limited as it relies on bulk tissue analysis. Consequently, rare abnormalities, such as those in a subgroup of cells within premalignant lesions, may be missed. In contrast, fluorescence *in-situ* hybridisation (FISH) allows nucleus-by-nucleus analysis of absolute copy number for any given chromosome-specific probe and is therefore more sensitive than CGH which detects chromosomal losses or gains relative to the overall genome of interest.

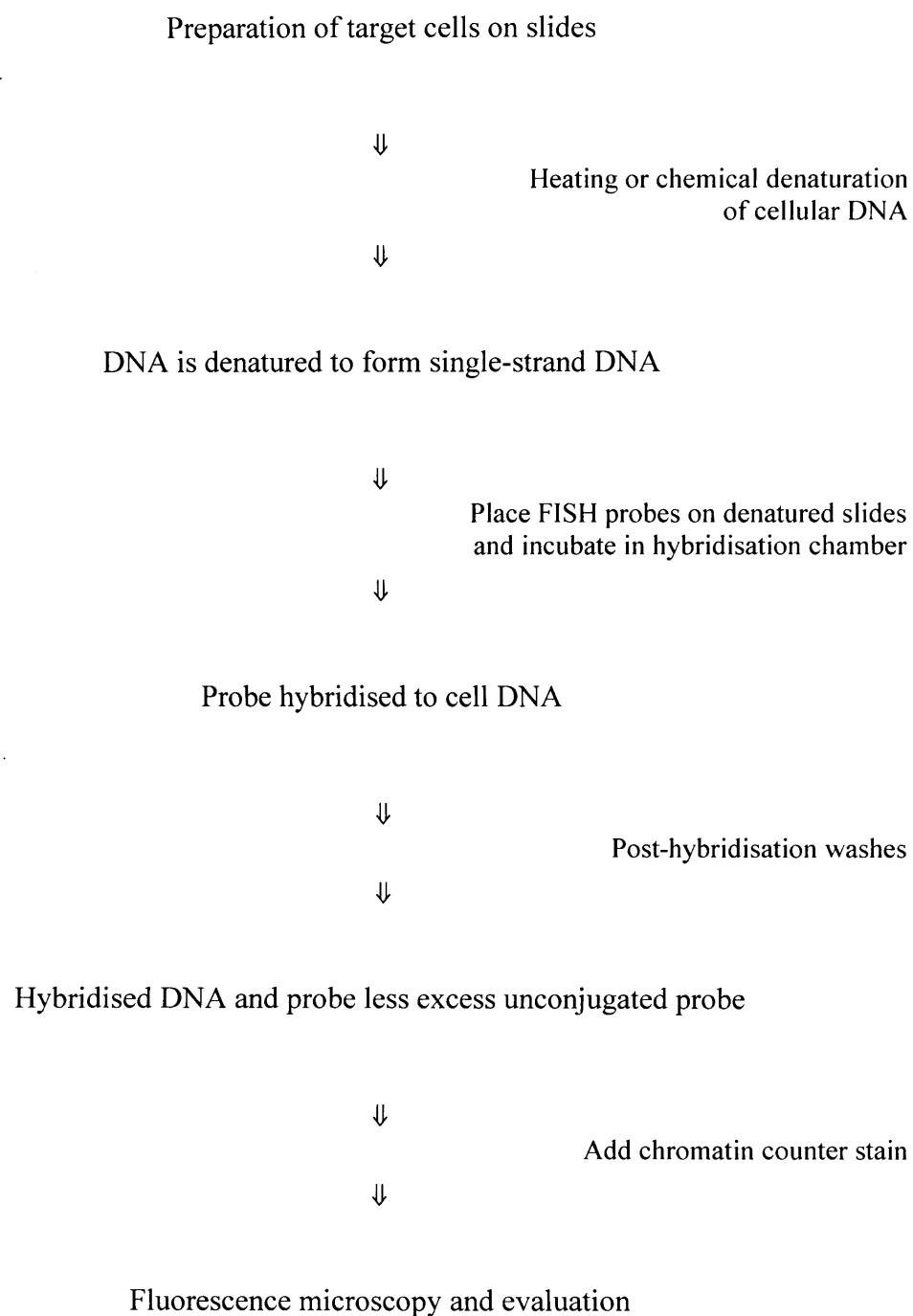
The *in-situ* hybridisation technique was originally described in 1969 (Gall and Pardue, 1969; John *et al*, 1969) and allows the identification of nucleic acid sequences within cells. A small DNA probe complementary to a section of target DNA is labelled and hybridised to tumour samples, the labelled probe binding to its counterpart in the tumour DNA. When *in-situ* hybridisation was first described, radioisotopes were the only labels available for nucleic acids. Subsequently, the development of fluorescent labels has replaced radioisotopes (Landegent *et al*, 1984). The *in-situ* hybridisation technique has been renamed fluorescence *in-situ* hybridisation (FISH) and has several advantages over *in-situ* hybridisation with radioisotopes, such as increased sensitivity, stability, safety and ease of use. The probe length for optimal hybridisation is 200-800 base pairs (Breneman *et al*, 1993). Probes exceeding 1000 base pairs and less than 200 base pairs are less efficient. If the probe concentration is too high non-specific signals are increased whereas if the probe concentration is too low the signals are insufficient for detection. In order to detect bound probes reporter macromolecules such as avidin and antibodies can be conjugated with fluorochromes. These compounds are referred to as indirectly labelled probes and have the ability to amplify signals. Directly labelled probes are those in which the fluorochrome is covalently attached to the DNA. The main advantages of directly labelled probes are a greater resolution and an increased signal to noise ratio. Numerical abnormalities of cell chromosomes can be detected with either whole chromosome probes in metaphase or with centromeric probes in interphase. Probes for the detection of autosomes would ordinarily be expected to produce two signals within a cell and an alteration in chromosomal number will be reflected by a similar alteration in the number of each signal visualised on fluorescence microscopy.

A typical FISH technique is summarised in Figure 1.5. In brief, the DNA within the nuclei of target cells on slides is first denatured chemically or with heat. The probes are placed on the denatured slides and incubated in a moist chamber overnight or for several days (depending on the nature of the probes). Within the hybridisation reaction four types of heteroduplex hybrids are formed: target to target; probe to probe; target to probe; and non-specific. Probe to probe and target to target hybrids, although specific bonds, are not detected and are washed from the slide. Excess probe which has not formed complementary base pairs with the cell DNA is washed from the slide and a counter stain added which is taken up by chromatin and aids localisation of probes within a nucleus. Fluorescence microscopy and evaluation of the slide is then possible.

FISH combines the resolution of molecular biology techniques to classical cytogenetics allowing precise localisation of nucleic acid sequences in either fresh tissues or archival material and can detect most of the structural and all of the numerical rearrangements present in a cell. FISH, which is a particularly sensitive technique as it is able to define changes at a cellular level, has been instrumental in the detection of a large number of structural and numerical chromosomal abnormalities involved in carcinogenesis (Patel *et al*, 2000) and is now also used routinely in clinical investigation (King *et al*, 2000).

FISH has been used to investigate genetic abnormalities in several premalignant tissues and has provided valuable information on early chromosomal changes which may be associated with tumourigenesis (Kurtycz *et al*, 1996; Persons *et al*, 1998; Ai *et al*, 2001). Therefore, FISH is ideally suited to detect early genetic

Figure 1.5. The fluorescence *in-situ* hybridisation technique.



changes at the enterovesical anastomosis) in patients who had undergone a clam ileocystoplasty.

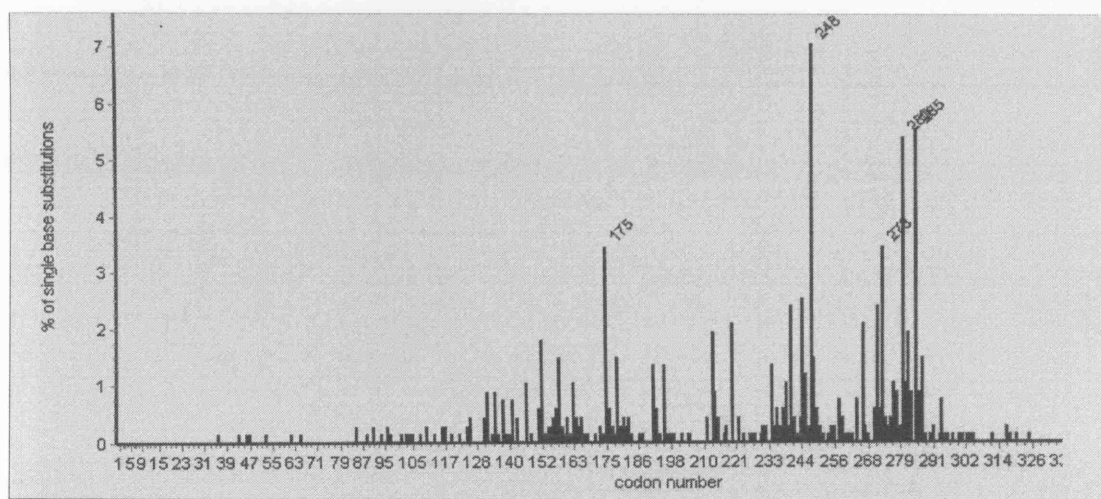
An alteration in the p53 gene is the most commonly described cancer-related genetic change and p53 mutations are found in almost fifty percent of all human tumours (Carson and Lois, 1995). It is not yet clear whether cancers occurring within an enterocystoplasty are primarily of bowel or bladder origin or are a separate entity. The p53 gene is altered in fifty to sixty percent of all transitional and squamous cell carcinomas of the bladder (Reznikoff *et al*, 2000) and up to seventy-five percent of colon adenocarcinomas (Hoops and Traber, 1997). Furthermore, there is a clear correlation between the presence of p53 mutations and the tumour stage and grade in bladder tumours (Reznikoff *et al*, 1996). Thus, a high frequency of p53 mutations has been detected in invasive bladder cancer but rarely in superficial, low grade tumours (Sidransky *et al*, 1991; Fujimoto *et al*, 1992). However, mutations of the p53 gene have been identified as an important early event in the development of carcinoma *in-situ* (Spruck *et al*, 1994), a condition which frequently progresses to invasive bladder cancer.

The p53 gene is located on the short arm of chromosome 17 (17p). Seventy percent of colorectal adenomas demonstrate loss of heterozygosity of 17p (Nagase and Nakamura, 1993). Sequence analysis of p53 in neoplasms with allelic loss on 17p often demonstrate mutations in the remaining allele implicating p53 as the major target tumour-suppressor gene in this region (Baker *et al*, 1989). These observations are supported by the finding that between fifty and seventy-five percent of colon adenocarcinomas have lost the function of both alleles of the p53 gene (Hoops and Traber, 1997). We are not aware of any study which has looked for mutations of any

gene in cancers within an enterocystoplasty. Since the frequency of p53 mutations in human tumours, and particularly the frequency of p53 mutations in both bladder and bowel tumours is high, the p53 gene would appear to be a suitable gene for study into the development of tumours in human enterocystoplasties.

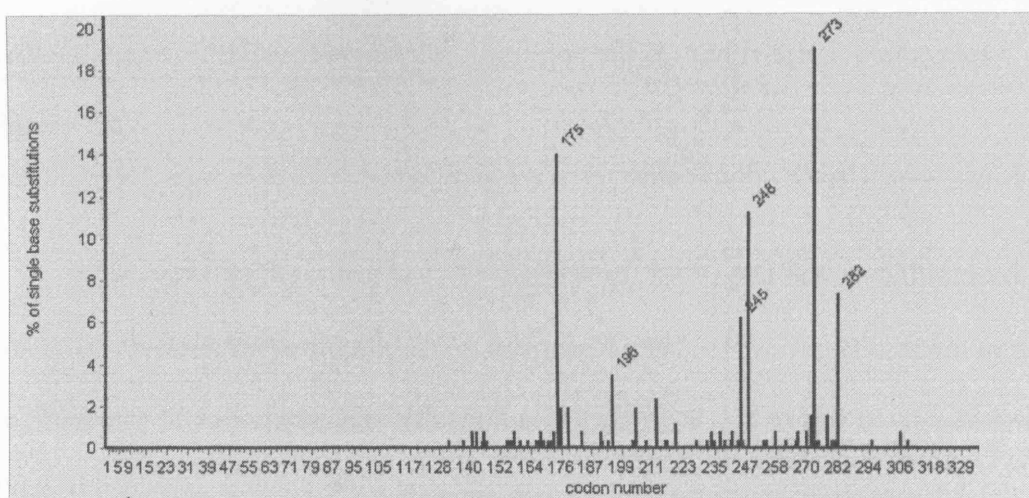
The TP53 mutation database (www.iarc.fr) is the largest compendium of disease related human somatic mutations (Hollstein *et al*, 1994). The database contains information concerning the frequency of p53 mutations at each codon of the p53 gene. Some codons appear in the database more frequently than others do and are so-called hotspot mutations. Hotspot mutations in cancers may represent protein alterations that provide a selective growth advantage to the cell and are more likely to result in tumourigenesis (Walker *et al*, 1999). Differential frequencies of p53 gene mutations are described in different cancer types indicating that there is no common underlying mechanism for p53 mutagenesis in different tissue types. The mutation types and their distribution (mutation spectrum) in the p53 gene differ between cancers. The codon distribution for mutations described in transitional cell carcinoma of the bladder and adenocarcinoma of the colon are shown in Figures 1.6 and 1.7. The highest concentrations of mutations within all described cancers are within codons 175, 196, 213, 245, 248, 249, 273 and 282 (Jenkins *et al*, 2001). The location of hotspot mutations usually denotes a functionally critical amino acid (Walker *et al*, 1999). Other factors contributing to hotspot mutations include a high susceptibility of a codon to mutation by particular carcinogens and slow DNA repair rates (Tornaletti and Pfeiffer, 1994). Hotspot codons coding for amino acid residues within the p53 protein are those areas which contact DNA and many of these residues form the core of the folded protein (Walker *et al*, 1999).

Figure 1.6. Codon distribution for mutations described in the p53 tumour suppressor gene in transitional carcinoma of the bladder (TP53 mutation database, www.iarc.fr).



DNA is constructed from a combination of the four nucleotides adenine, cytosine, guanine and thymine. Codons are sequences of three adjacent nucleotides and each codon specifies a single amino-acid in the protein product of DNA. Mutations are changes in the nucleotide sequence of DNA. This figure demonstrates the distribution of mutations described for the p53 gene in transitional carcinoma of the bladder. The highest reported incidence of p53 mutations in transitional cell carcinoma of the bladder is in codons 175, 248, 273, 280 and 285.

Figure 1.7. Codon distribution for mutations described in the p53 tumour suppressor gene in adenocarcinoma of the colon (TP53 mutation database, www.iarc.fr).



DNA is constructed from a combination of the four nucleotides adenine, cytosine, guanine and thymine. Codons are sequences of three adjacent nucleotides and each codon specifies a single amino-acid in the protein product of DNA. Mutations are changes in the nucleotide sequence of DNA. This figure demonstrates the distribution of mutations described for the p53 gene in adenocarcinoma of the colon. The highest reported incidence of p53 mutations in adenocarcinomas of the colon is in codons 175, 196, 245, 248, 273 and 282.

The Restriction Site Mutation (RSM) assay, first described in 1990, allows the detection of rare mutations in the presence of a vast excess of background unmutated wild-type sequences and is therefore an ideal technique for identifying early mutations present in premalignant tissue (Parry *et al*, 1990). The RSM assay is employed in cancer research to study mutation prevalence at key hotspot codons of tumour related genes which fall within restriction enzyme sites which accompany and possibly cause tumour progression. The RSM assay combines the research methodologies of the polymerase chain reaction (PCR) and restriction enzymes (Figure 1.8).

PCR was first described in 1985 (Saiki *et al*, 1985) and has revolutionised molecular research by permitting rapid analysis of DNA. However, it was not until the discovery of a thermostable endonuclease (Saiki *et al*, 1988) and the development of thermal cycling technology which enabled the automation of PCR, that the technique became widely used.

PCR allows the amplification of specific DNA sequences (Figure 1.9). The reaction relies on the annealing of two short oligonucleotide primers, each complementary to the flanking DNA sequence of the region of DNA to be amplified. These primers are extended by the action of *Taq* polymerase, a thermostable DNA endonuclease. The resulting double-stranded DNA consisting of one template strand and one extended strand is denatured at high temperature. This allows the primers to anneal once more. Annealing is followed by further extension by *Taq* polymerase. Denaturation, annealing and extension are carried out in turn for multiple cycles resulting in a logarithmic amplification of the target DNA sequence.

Figure 1.8. The restriction site mutation assay.

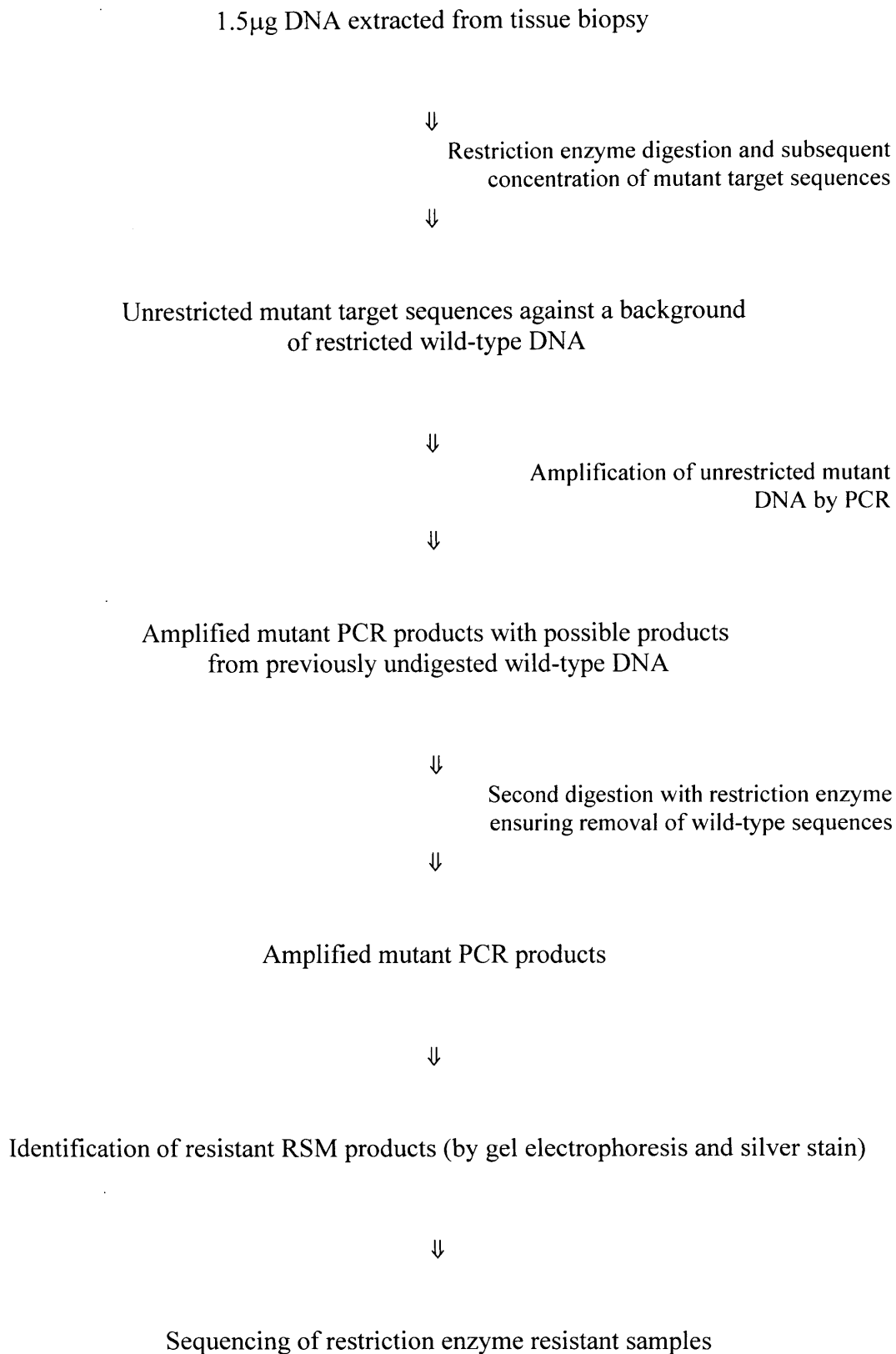
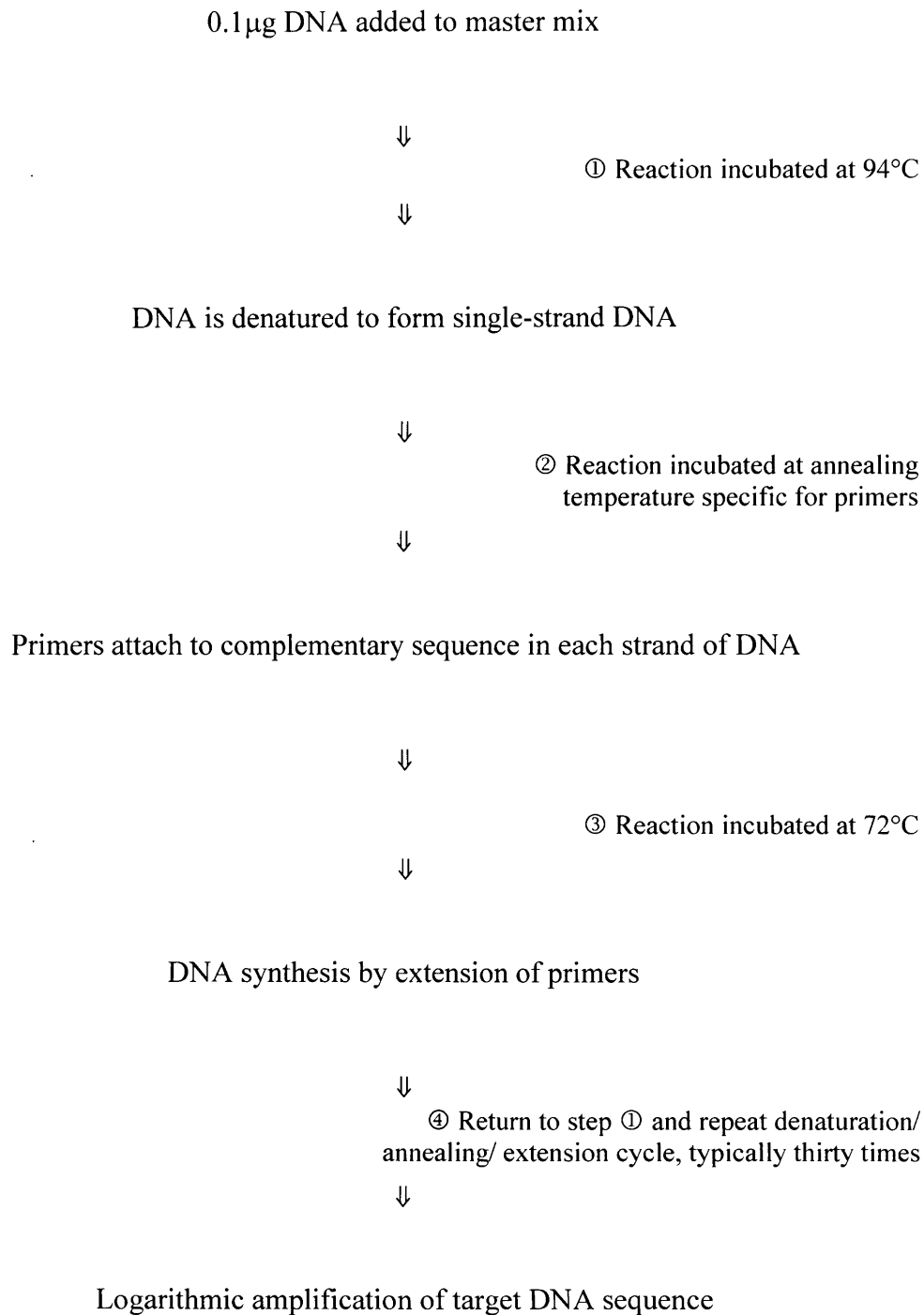


Figure 1.9. The polymerase chain reaction.



Type II restriction enzymes are bacterial endonucleases that have the ability to recognise specific DNA sequences and cleave DNA at such sites. Mutations occurring at restriction sites remove the ability of restriction enzymes to recognise the DNA sequence. The use of restriction enzymes can therefore provide a method of screening large numbers of tissue samples for the presence of tumour related mutations. Since restriction enzymes only recognise specific DNA sequences, batteries of restriction enzymes are generally employed to cover as many of the important hotspot codons of tumour-related genes as possible (Jenkins *et al*, 2002).

The restriction enzymes used have recognition sequences which are present at codons with a high reported mutation frequency. This digestion step cleaves the DNA of any non-mutated sequences so that subsequent PCR is unable to amplify the target DNA. However, any rare mutated sequences that remain intact and are not recognised by the restriction enzyme are amplified by subsequent PCR. The detection sensitivity of this technique depends heavily upon, and is limited by, the efficient removal of unmutated wild-type sequences (Parry *et al*, 1990). Therefore, a second digestion is performed after PCR amplification to remove any trace of wild-type DNA that may be present after the initial digestion. Sequencing the PCR products obtained allows the mutations to be characterised and also eliminates the possibility of false positive results due to amplification of undigested wild-type DNA. P53 mutations highlighted by RSM analysis at the bladder-bowel interface of patients who have undergone a clam ileocystoplasty would indicate instability at the enterovesical anastomosis and may identify those patients at an increased risk of carcinoma formation.

In the RSM assay, targeted restriction sites of DNA from biopsy material are digested by restriction enzymes. After digestion the remaining uncut mutated DNA is amplified by PCR using primers flanking the target codon. Normal DNA will have undergone digestion and cannot undergo amplification by PCR and hence, only mutated sequences are amplified. This allows the very rare mutated sequences to be amplified from an excess of normal wild-type sequences. The detection sensitivity of this technique depends heavily upon, and is limited by, the efficient removal of unmutated wild-type sequences (Parry *et al*, 1990). Therefore, a further restriction enzyme digestion of the RSM products is performed to remove any amplified products containing wild-type restriction sites. The undigested, mutated PCR products obtained are then sequenced to characterise any detected mutations.

The RSM assay is limited to analysing the four to six bases which make up the enzyme recognition sequence but by studying multiple restriction sites, it is possible to increase the target size. Five of the eight main p53 mutation hotspots detected in cancers contain restriction enzyme sites, thus enabling RSM analysis to detect rare mutations at these codons namely: 175 (*Hha* I, GCGC), 213 (*Taq* I, TCGA), 248 (*Msp* I, CCGG), 249/ 250 (*Hae* III, GGCC) and 282 (*Msp* I, CCGG). The RSM assay cannot investigate mutations at the other three main hotspots (codons 196, 245 and 273) as there are no known restriction enzymes with recognition sites specific to these codons (Jenkins *et al*, 2001).

The sensitivity of the RSM assay is determined by the amount of deoxyribonucleic acid (DNA) that can be screened in each analysis. The optimum amount of DNA that supports amplification is 1 to 2 µg (McPherson *et al*, 1994), representing 3 to 6 x 10⁵ copies of the genome. It has been shown that about ten

copies of a mutation resistant to enzyme digestion are required to produce a visible band on a gel after RSM analysis (Steingrimsdottir *et al*, 1996, Jenkins *et al*, 2001). In practice, RSM analyses have been shown to detect mutations at a frequency of approximately 10^{-4} , which is one mutant in 10,000 wild type sequences (Jenkins *et al*, 1999). This sensitivity can be increased by performing analyses at multiple restriction sites, thereby increasing the chances of rare mutations being detected.

Any technique that uses PCR should assess the level of *Taq* polymerase error which could affect the results. However it is felt that the contribution of *Taq* polymerase error is negligible in the RSM assay (Jenkins *et al*, 1999). High fidelity PCR regimes are used for PCR (McPherson *et al*, 1994) and *Taq* polymerase is a high fidelity enzyme. In addition, at least ten identical mutations are required to obtain an identifiable PCR product with the RSM assay (Steingrimsdottir *et al*, 1996; Jenkins *et al*, 2001). Furthermore, *Taq* polymerase error does not produce transversions (Keohavong and Thilly, 1988) and mutations detected in previous RSM experiments have shown a large proportion of transversion events (Jenkins *et al*, 1999).

The RSM assay is a highly sensitive technique suitable for the detection of rare mutations in premalignant tissue. In principle, the RSM methodology may be used to study rare mutational events in any organ of any species, for which the DNA sequence information is available. The technique has been optimised and validated through the detection of rare mutations induced in experimental animals and in cultured cells by the exposure of known mutagens (Perwez Hussain *et al*, 1994a; Perwez Hussain *et al*, 1994b; Jenkins *et al*, 1997; Jenkins *et al*, 1998). Furthermore, the RSM assay has been used to detect the presence of mutations in key tumour

related genes in clinical samples. Early p53 mutations have been reported in inflammatory colon tissue (Ambs *et al*, 1999, Perwez Hussain *et al*, 2000), Barrett's oesophagus (Jenkins *et al*, 2003) and inflammatory gastric tissue (Morgan *et al*, 2003) using RSM analysis suggesting that mutations in this suppressor gene may be early, initiating events in the pathogenesis of oesophageal, gastric and inflammatory bowel cancer. Consequently the RSM assay has the potential to confirm and characterise possible early genetic alterations in any premalignant tissue and is ideally suited for the detection of early p53 mutation present at the anastomosis in clam enterocystoplasties.

1.5. Summary and aims.

There is a considerable body of evidence to suggest that there is a small, but increased risk of cancers arising in enterocystoplasties (Chapters 1.3 and 1.4):

- The number of reported cases of carcinoma formation within enterocystoplasties has increased
- A large number of tumours forming within enterocystoplasties are adenocarcinomas, which are rarely reported in the bladder or small bowel
- The histology, anastomotic localisation and latency of clam cancers are similar to those of tumours occurring in ureterosigmoidostomies, an operation which is recognised to increase the risk of malignancy
- The histology and location (at the anastomosis) of clam tumours are similar to tumours that occur in animals with an enterocystoplasty
- Many tumours are located adjacent to the enterovesical anastomosis suggesting that inflammation, abnormal stromal–epithelial signalling or altered cell metabolism may contribute tumourigenesis
- The enterovesical anastomosis in clam enterocystoplasties are frequently inflamed at cystoscopy
- Nitrosamines are potent carcinogens. Elevated levels of nitrosamines are present in the urine of patients who have undergone an enterocystoplasty, particularly if the cystoplasty is inflamed
- Chromosomal changes have been detected by CGH at the enterovesical anastomosis in some patients with an enterocystoplasty.

These observations suggest that the anastomosis following an enterocystoplasty is inherently genetically unstable

Since tumours arising within enterocystoplasties are aggressive and are frequently life-threatening, there is a need to identify which patients are most at risk from tumour formation.

Recent studies using comparative genomic hybridisation (CGH) in patients with a clam ileocystoplasty, identified abnormalities of chromosomes 9 and 18 in biopsies obtained adjacent to the enterovesical anastomosis, observations which suggest that the urothelium is genetically unstable (Appanna *et al*, 2000, Appanna, 2004). Amplifications of chromosomes 8p and 21q have been reported in two clam cancers using CGH suggesting that instability of these chromosomes may also be important in the development of cancers within augmentation cystoplasties (Appanna *et al*, 2000, Appanna, 2004). Although CGH is a useful cytogenetic technique that assesses chromosomal alterations on a global scale, its sensitivity is limited as it relies on bulk tissue analysis. Consequently, rare abnormalities, such as those in a subgroup of cells within premalignant lesions, may be missed. In contrast, fluorescence *in-situ* hybridisation (FISH) allows nucleus-by-nucleus analysis of absolute copy number for any given chromosome-specific probe and is therefore more sensitive than CGH which detects chromosomal losses or gains relative to the overall genome of interest. FISH has been used to investigate genetic abnormalities in several premalignant tissues and has provided valuable information on early chromosomal changes which may be associated with tumourigenesis (Kurtycz *et al*, 1996; Persons *et al*, 1998; Ai *et al*, 2001). Therefore, the purpose of the first part of this thesis was to further investigate abnormalities involving chromosomes 8, 9 and 18 using FISH (an appropriate FISH probe for chromosome 21 alone is not currently commercially available) in patients who had undergone a clam ileocystoplasty to detect early genetic changes at the enterovesical anastomosis. FISH was also

performed on biopsies taken distant from the anastomosis (controls) in patients who had undergone an enterocystoplasty. Material from a 'clam' tumour was also investigated using FISH.

We are not aware of any study which has looked for mutations of any gene in cancers within an enterocystoplasty. Since the frequency of p53 mutations in human tumours is high we investigated the p53 gene in human enterocystoplasties. The restriction site mutation (RSM) assay is a technique that is ideally suited for detecting rare mutations in premalignant tissue. Previous studies have demonstrated a high incidences of early p53 mutations in atrophic gastritis (Morgan *et al*, 2003) inflammatory colon tissue (Ambs *et al*, 1999; Perwez Hussain *et al*, 2000) and Barrett's oesophagus (Jenkins *et al*, 2003) suggestive of premalignant change. The aim of part of this thesis was therefore to determine whether early p53 mutations are present in biopsies taken from the enterovesical anastomosis in patients who have undergone a clam enterocystoplasty. The presence of p53 mutations in biopsies taken distant from the anastomosis (controls) in patients who had undergone an enterocystoplasty was also investigated. The presence of p53 mutations was also investigated in material from a 'clam' tumour.

Therefore, the aims of the present study were to:

1. Determine the chromosomal abnormalities in individual cells at and distant from the enterovesical anastomosis in patients with a clam ileocystoplasty.
2. Whether p53 mutations are present in biopsies taken at and distant from the enterovesical anastomosis in patients with a clam ileocystoplasty.

1.6. Null hypothesis.

The enterovesical anastomosis in patients with a clam ileocystoplasty is genetically stable and does not predispose to tumour formation.

2. Materials and Methods.

2.1. General.

Unless otherwise stated, all chemical reagents used in the experiments were purchased from Sigma (Sigma Chemical Company, Poole, Dorset, UK) at the highest available purity. Solutions were autoclaved or prepared using filter sterilised and deionised, purified water (Milli Q water purification system, Millipore, Watford, UK). Disposable gloves and laboratory coats were worn for all experiments. All reagents were handled and disposed of in accordance with locally agreed safety protocols. Materials and Methods specific to individual chapters are addressed in the relevant sections.

2.2. Collection of biopsy samples.

Ethical consent for this work was granted by Bro Taf Local Research Ethics Committee prior to the start of the study. Patients who wished to participate in the study gave written and verbal informed consent. It is standard practice in our unit that patients who have undergone a clam enterocystoplasty are followed-up yearly, by performing rigid cystoscopy and biopsy of the urothelium at the enterovesical anastomosis. In addition to the usual biopsy for histology, where possible 3 further biopsies were taken from the enterovesical anastomosis and 3 from the native bladder remnant distant from the enterovesical anastomosis. For the purpose of this study, all biopsies were taken by Mr. T.P. Stephenson. When touch sample preparations were required these were performed immediately (Chapter 2.4) and the biopsies stored dry in a freezer at -20°C.

2.3. Patient population used for cytogenetic studies.

Successful touch sample preparations were obtained from 15 patients (Chapter 2.4). In 7 patients clam enterocystoplasty had been performed for the treatment of a neuropathic bladder and in 8 for treatment of an overactive non-neuropathic bladder, 4 of whom had a lifelong unstable bladder. No patient received a clam enterocystoplasty for treatment of tuberculous cystitis. All patients were augmented with ileum. 8 patients were male and 7 were female. The average age of the patients at the time of biopsy was 35.9 years (median 31 years), with a range of 21 to 62 years. The average time since undergoing clam enterocystoplasty was 12.5 years (median 12 years), with a range of 6 to 18 years.

2.4. Touch sample preparations.

Touch sample preparations were prepared with fresh biopsy material. Attempts to obtain touch preparations with frozen biopsies were unsuccessful. Glass slides were cleaned with absolute ethanol and air-dried. A diamond-tipped glass marking pen was used to mark an area equivalent to a cover slip on the slide. Using forceps, bladder biopsies were touched repeatedly onto the slide until the marked area had been covered taking care not to smear the sample across the slide. The cells were fixed onto the glass slides, air-dried for 10 minutes, immersed in methanol/ acetic acid 3:1 mix (Fisher Scientific, Loughborough, Leicestershire, UK) for 20 minutes, immersed in fresh methanol/ acetic acid 3:1 mix for a further 20 minutes and air dried for 30 minutes. The slides were visualised under a light microscope to assess their quality and successful touch preparations were stored in a freezer at -20°C.

2.5. Pepsin digestion of touch sample preparations.

Epithelial cells have a tough, keratinised cell membrane, which makes penetration of fluorescent probes difficult. Pepsin removes cellular proteins such as keratin in the cellular and nuclear membrane facilitating probe penetration. Prepared slides and a solution of pepsin (300 µg pepsin, 100 ml 0.01 M HCl, pH 2.8) were incubated at 37°C for 10 minutes. Pepsin solution was placed on the slides liberally covering the area of the touch sample preparation and incubated at 37°C for exactly 7 minutes. The pepsin was removed by immersing slides in phosphate buffered saline (PBS) solution (0.14 M NaCl, 25 µM KCl, 8 µM Na₂PO₄, 1.8 µM KH₂PO₄, pH 7.4) for 5 minutes at room temperature after which they were immersed in PBS containing magnesium chloride (0.14 M NaCl, 25 µM KCl, 8 µM Na₂PO₄, 1.8 µM KH₂PO₄, 50 µM MgCl₂) at room temperature for a further 5 minutes to inactivate any residual pepsin and then air-dried.

2.6. Fluorescence *in-situ* hybridisation.

2.6.1. Dehydration of slides.

Touch sample preparations that had undergone pepsin digestion (Chapter 2.5) were dehydrated by sequential immersion in 70%, 80% and 95% ethanol, (each immersion lasting for 2 minutes) and then air-dried.

2.6.2. Denaturation.

For all experiments orange, green and aqua fluorescent centromeric probes were used to identify chromosomes 8, 9 and 18 respectively (Vysis, Nottingham, UK). The fluorescent probe for chromosome 8 was part of a commercial kit for clinical testing, and was already added to hybridisation buffer.

8 µl of chromosome 8 probe/ hybridisation mixture was placed in an ependorff tube and 1 µl of the probe for chromosome 9 and 1 µl of the probe for chromosome 18 added and mixed with a pipette. The resulting solution was placed on the target area of each slide and a cover slip applied. The edges of the cover slip were sealed by the application of a rubber solution. The fluorescent probes and target DNA were denatured by placing the slides on a hot plate with a surface temperature of 75°C for exactly 2 minutes.

2.6.3. Hybridisation.

Slides were hybridised in a light proof humidified chamber at 37°C for 16 hours.

2.6.4. Post-hybridisation washes.

The cover slip and rubber cement were removed, the slides immersed in 0.4x standard saline citrate (SSC) / 0.3% Nonidet-40 (NP-40) solution (0.06 M NaCl, 0.006 M trisodium citrate, 0.3% NP-40 (Vysis, Nottingham, UK), pH 7.5) at 73°C for exactly 2 minutes, agitated for 3 seconds on immersion, immersed in 2x SSC/ 0.1% NP-40 (0.3 M NaCl, 0.03 M trisodium citrate, 0.1% NP-40, pH 7.0) solution at room temperature for 30 seconds and air-dried.

2.6.5. Fluorescence microscopy and evaluation.

10 µl 4', 6'-diamidino-2-phenylindole hydrochloride (DAPI II) (Vysis) was placed on the target area of each slide, a cover slip applied and placed in a refrigerator at 4°C for 10 minutes to allow the colour to develop. Fluorescent microscopy was performed using an Olympus BX50 microscope equipped with single pass filters (Olympus, Southall, Middlesex, UK) to visualise orange, green and

aqua fluorescent probes. Images were captured under fluorescence microscopy using Macprobe v4.3 (PowerGene, Newcastle upon Tyne, UK) image analysis software. 200 nuclei were scored for each sample. If slides were of insufficient quality to score 200 nuclei, FISH was performed on further slides and cells scored until the required number had been obtained. Normal values were obtained by analysing the data obtained from the control specimens. The cut-off criteria used in categorising results as normal versus abnormal were defined as three standard deviations from the mean of the control values and calculated for both monosomy and trisomy for each of the chromosomes studied. The use of such a threshold is consistent with the analysis performed by other researchers (Huang *et al*, 1999; Botti *et al*, 2000; Debiec-Rychter *et al*, 2001). Chromosomal counts exceeding the normal range were considered abnormal.

2.7. Patient population in restriction site mutation assay (RSM) studies.

DNA was obtained from biopsies from a 38 patients for RSM analysis. Mr Timson Appanna donated the DNA for 9 of these samples that had been previously obtained for research into carcinogenesis in clam ileocystoplasties under the same ethical approval covering this work. A clam enterocystoplasty was performed for the treatment of a neuropathic bladder in 20 patients and for the management of an overactive non-neuropathic bladder in 17 patients, 10 of which had a lifelong unstable bladder. One further patient had undergone a clam enterocystoplasty for treatment of tuberculous cystitis. All patients were augmented with ileum. 16 patients were male and 22 were female. The mean age of patients at the time of their biopsy was 35.7 years (median 30 years), with a range of 18 to 65 years. The average time since undergoing clam enterocystoplasty was 12.4 years (median 11.5 years), with a range of 4 to 41 years.

2.8. DNA extraction from tissue.

DNA was extracted using a Stratagene DNA Extraction Kit (Stratagene, Cambridge, UK). The DNA from one anastomotic biopsy and one control biopsy taken from the native bladder remnant was analysed for each patient. A single bladder biopsy was added to a labelled sterile 1.5 millilitre (ml) ependorff tube containing 270 microlitres (μ l) of lysis buffer (10 mM Tris-HCl, pH 8.2, 400 mM NaCl, 2 mM EDTA). 5 μ l of 225 mg/ml pronase (Stratagene, Cambridge, UK) was added and the solution shaken at 220 revolutions per minute (rpm) at 37°C overnight. After lysis the samples were chilled on ice for 10 minutes the protein precipitated by the addition of 90 μ l of 6 M NaCl, the solution inverted several times to ensure adequate mixing and stored on ice for a further 5 minutes. The protein precipitate was pelleted by centrifugation for 15 minutes at 3400 rpm in an Eppendorf 5415D centrifuge. The supernatant containing nucleic acids was transferred to a sterile 1ml ependorff tube. If any of the protein precipitate remained in the supernatant further centrifugation for 15 minutes at 3400 rpm was performed and the supernatant obtained transferred to another sterile 1 ml ependorff tube. 3 μ l of 10 mg/ml RNase (Stratagene, Cambridge, UK) was added and the solution incubated at 37°C for 15 minutes. DNA was precipitated by adding an equal volume of isopropanolol that had been stored at -20°C. The solution was stored at -20°C for at least 1 hour to allow optimal precipitation of DNA which was pelleted by centrifugation at 3400 rpm for 15 minutes, washed with 70% ethanol, centrifuged for a further 15 minutes at 3400 rpm, the ethanol removed using a pipette and the DNA dried at 37°C overnight to remove residual ethanol by evaporation. The DNA was resuspended in 100 μ l of deionised water and stored at -20°C.

2.9. Quantification of DNA.

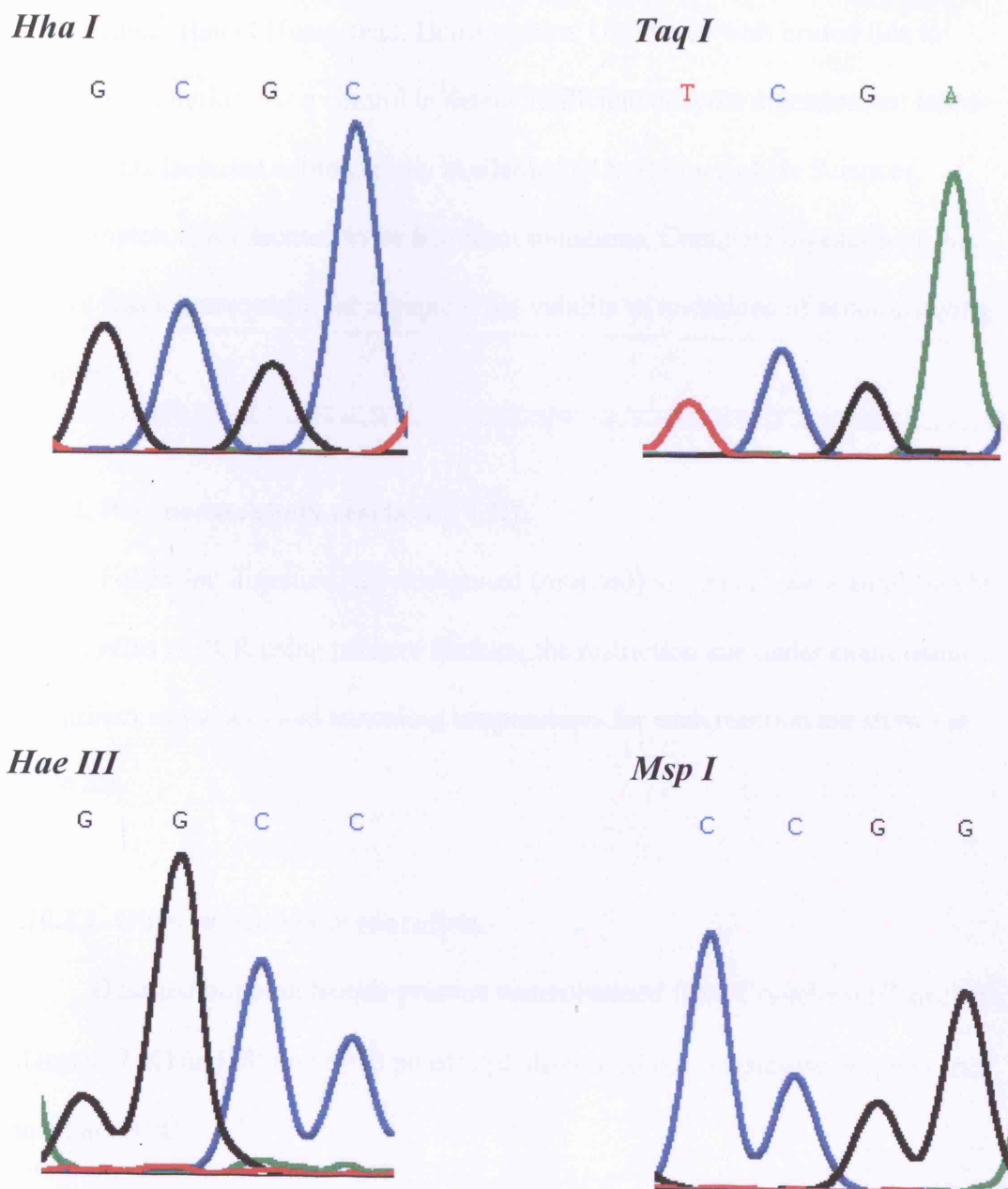
A sample of DNA was diluted with filtered sterilised water and added to a quartz cuvette. DNA concentrations were analysed using a Beckman DU-65 spectrophotometer (Beckman Coulter, High Wycombe, Buckinghamshire, UK) and the DNA concentration calculated by multiplying the measured concentration by the dilution factor. DNA was diluted to a working concentration of 100 ng/μl and stored at -20°C. The quality of extracted DNA was further analysed by performing a Polymerase Chain Reaction (PCR) on each sample (Chapter 2.10.2) and running the product obtained on a polyacrylamide gel (Chapter 2.11). The DNA was considered of sufficient quality for analysis when good visualisation of PCR products was present.

2.10. Restriction site mutation (RSM) assay.

2.10.1. Restriction enzyme digestion of genomic DNA.

The DNA from the enterovesical anastomosis and control specimen from each patient was digested by restriction enzymes. Restriction enzyme digestion (Promega Life Sciences, Southampton, UK) was carried out by incubating 1.5 μg DNA (100 ng/μl) with 2 μl of 10x *Taq* Polymerase buffer (100 mM Tris-HCl, pH 8.8, 500 mM KCl, 1% Triton[®] X-100 (Promega Life Sciences, Southampton, UK)), 1 μl MgCl₂ (1.25 mM) and 2 μl restriction enzyme (10 units/μl). A separate reaction was required for each restriction site studied. The RSM assay was used to study five known hotspots using the restriction enzymes (Promega Life Sciences, Southampton, UK) *Hha* I (codon 175), *Taq* I (codon 213), *Hae* III (codon 249/250) and *Msp* I (codons 248 and 282) (Figure 2.1). The experimental conditions for each enzyme

Figure 2.1. Sequencing data showing recognition sites in wild-type DNA for the restriction enzymes used to perform restriction site mutation (RSM) analysis.



DNA is constructed from a combination of the four nucleotides adenine (A), cytosine (C), guanine (G) and thymine (T). DNA sequencing determines the sequence of nucleotides in a length of DNA. The sequencer detects signals from nucleotides labelled with four different fluorescent dyes. These signals are then plotted on a sequence trace. Recognition sites are the specific nucleotide sequence to which a restriction enzyme binds prior to cutting DNA at that site. Examples of DNA traces obtained from the recognition sites for each of the four restriction enzymes used in RSM analysis are shown.

digestion are shown in Table 2.1. Restriction enzyme digestions were carried out for set periods of time according to the manufacturer's recommendations in 0.2 ml thin-walled tubes using a DNA engine thermal cycler (MJ Research PTC-2000 Peltier, MJ Research, Hemel Hempstead, Hertfordshire, UK) fitted with heated lids to prevent evaporation. As a control to detect inefficient enzyme digestion, all RSM experiments included commercially available DNA (Promega Life Sciences, Southampton, UK) deemed to be free from mutations. Complete digestion of this control was a prerequisite for accepting the validity of mutations of accompanying samples.

2.10.2. Polymerase chain reaction (PCR).

Following digestion, the undigested (mutated) sequences were amplified by thirty cycles of PCR using primers flanking the restriction site under examination. The primer sequences and annealing temperatures for each reaction are shown in Table 2.2.

2.10.2.1. Oligonucleotide preparation.

Desalted oligonucleotide primers were obtained from Cruachem (Cruachem, Glasgow, UK) and diluted to 10 pmoles/ μ l, dispensed into single-use aliquots and stored at -20°C.

2.10.2.2. Prevention of PCR contamination.

Routine precautions were taken to reduce contamination of PCR products. PCR reactions were carried out in laminar flow hoods located in a separate laboratory to the one where DNA extraction and genetic manipulation were performed. A set of pipettes (Finnpipette, LabSystem, Leighton Buzzard, UK) were

Table 2.1. Properties of restriction enzymes used in RSM analysis.

Restriction enzyme	Recognition sequence	Host bacteria	Required Temperature	Time to complete digestion reaction
<i>Hha</i> I	GCG C C GCG	Haemophilus haemolyticus	37 °C	16 hours
<i>Taq</i> I	T CGA AGC T	<i>Thermus aquaticus</i>	65 °C	4 hours
<i>Hae</i> III	GG CC CC GG	<i>Haemophilus aegyptius</i>	37 °C	16 hours
<i>Msp</i> I	C CGG GGC C	<i>Moraxella species</i>	37 °C	16 hours - then add further 10 Units of <i>Msp</i> I to digestion reaction and incubate for further 2 hours

Table 2.2. Oligonucleotide primers used in PCR of DNA after digestion by restriction enzymes.

Codon	Restriction enzyme	Exon	Annealing temperature	PCR product size /base pairs	Oligonucleotide primers
175	<i>Hha</i> I	5	60 °C	71	Forward primer: d(CCGCGCCCATGGCATCT) Reverse primer: d(GCGCTCATGGTGGGG)
213	<i>Taq</i> I	6	65 °C	188	Forward primer: d(GTCCCAGGCCCTCTGATTCTC) Reverse primer: d(TAACCCCTCCTCCCAGAGACCCAG)
248	<i>Msp</i> I	7	60 °C	79	Forward primer: d(ATGTGTAAACAGTTCTCTGCATG) Reverse primer: d(CTGACCTGGAGTCTTCCAGTG)
249/250	<i>Hae</i> III	7	60 °C	79	Forward primer: d(ATGTGTAAACAGTTCTCTGCATG) Reverse primer: d(CTGACCTGGAGTCTTCCAGTG)
282	<i>Msp</i> I	8	60 °C	209	Forward primer: d(ACCTGATTTCCTTACTGCCTCTTGCTTC) Reverse primer: d(CTTGGTCTCTCCACCGCTCTTG)

kept for PCR use only and all pipette tips contained aeroguard filters (Alpha Laboratories, Eastleigh, Hampshire, UK). Laminar flow hoods, pipettes and tube racks were cleaned with 70% ethanol before use. The 0.2 ml tubes (Abgene, Epsom, UK) used for PCR were autoclaved prior to use and only opened inside the hoods. A master mix was prepared containing all the reagents of the reaction apart from DNA and aliquoted into individual tubes before the samples were added. A negative control (no template DNA) was included every time PCR was performed as a marker for contamination.

2.10.2.3. PCR protocol.

The PCR amplification was performed with 2.5 units of DNA *Taq* polymerase in thermo buffer (10 mM Tris-HCl, pH 8.8, 50 mM KCl, 0.1% Triton[®] X-100) along with 1.5 mM MgCl₂, 100 µM of each dNTP and 10 pmoles of each PCR primer. The final volume was adjusted to 50 µl with sterilised deionised water. PCR amplification was performed in a DNA engine thermal cycler (MJ Research PTC-2000 Peltier, MJ Research, Hemel Hempstead, Hertfordshire, UK). The reaction was initially incubated at 94°C for 2 minutes to completely denature the templates. Thermal cycling proceeded at 94°C for 30 seconds before continuing for 20 seconds at the annealing temperature specific to each pair of primers. Incubation for a further 20 seconds at 72°C allowed extension of the PCR products. The cycle was repeated many times to allow adequate amplification of PCR products. The annealing temperatures and cycle numbers for each experiment are specified in the relevant chapters.

2.10.3. Restriction enzyme digestion of PCR product.

After amplification, the PCR product was digested with the restriction enzyme under test to remove any wild-type DNA sequences that may have escaped initial digestion. 16 µl of PCR product was digested with 2 µl of 10x enzyme specific buffer (Promega Life Sciences, Southampton, UK) and 2 µl of restriction enzyme. Digestion was performed for set periods of time at the manufacturer's recommended temperature (Table 2.1) to ensure complete cleavage of wild-type DNA sequences.

2.10.4. PCR of products of restriction enzyme digestion.

One µl of the final RSM product was re-amplified by 12 cycles of PCR to ensure that undigested PCR products were present in much greater numbers for sequencing.

2.11. Visualisation of PCR product.

2.11.1. Polyacrylamide gel preparation.

The PCR products obtained were analysed using 6% polyacrylamide gels. A 6% solution for 4 gels was prepared using 8 ml of acrylamide (30% liquid acrylamide: bisacrylamide [37.5: 1], Severn Biotech, Kidderminster, Worcestershire, UK). 4.5 ml 10x TBE buffer (0.89 M Tris-Borate, pH 8.0, 0.89 M Boric acid, 0.02 M EDTA) and 32 ml of deionised water. The solution was vacuum degassed for 5 minutes before adding 45 µl of Temed (Gibco BRL, Paisley, Scotland, UK) and 220 µl of 10% ammonium persulphate solution (Gibco BRL, Paisley, Scotland, UK). The degassed solution was poured into 4 individual gel casters (Protean III gel system BIO-RAD, Hemel Hempstead, Hertfordshire, UK), 15 tooth combs inserted to form wells and the solution allowed to set at room temperature for 20 to 30 minutes.

2.11.2. Polyacrylamide gel electrophoresis.

Solutions comprised 3 µl of loading buffer (1% Bromophenol blue, 50% glycerol, 0.1 M EDTA, 1% lauryl sulphate) and 10 µl of PCR product. 10 µl of these solutions were loaded onto the gels together with a 100 base pair DNA molecular weight marker (Promega Life Sciences, Southampton, UK). 3 µl of the marker was made up with 3 µl of loading buffer and 7 µl of deionised water and 10 µl of the resulting solution loaded onto the gel. Electrophoresis was performed in 1x TBE buffer in vertical electrophoresis tanks (BIO-RAD, Hemel Hempstead, Hertfordshire, UK) at a constant power of 50W using a Pharmacia power pack (EPS 3500XL, Pharmacia, Sandwich, Kent, UK). Electrophoresis was carried out until the loading dye reached the bottom of the plate.

2.11.3. Silver staining of polyacrylamide gels.

Gels were stained in 0.1% silver nitrate for 5 minutes and washed twice in deionised water before being developed in 1.5% sodium hydroxide and 0.15% formaldehyde solution until PCR bands appeared.

2.11.4. Capture of silver stained gels.

Images of silver stained gels were captured using the GelDoc 2000 system (BIO-RAD, Hemel Hempstead, Hertfordshire, UK). Silver stained gels were illuminated using white light and the brightness and size manually adjusted to give the best image.

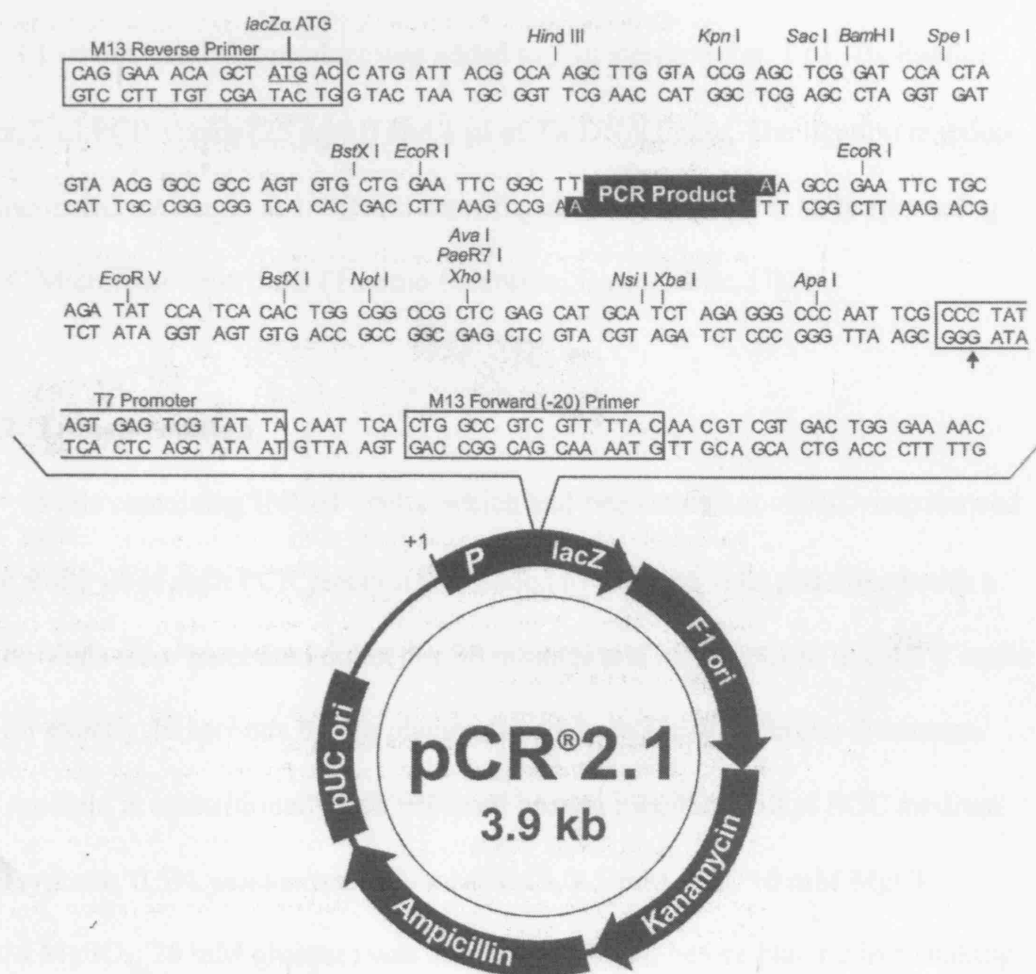
2.11.5. Purification of PCR products.

Purification of PCR products is required prior to sequencing. Purification of non-cloned PCR products was performed using a QIAprep PCR purification kit (Catalogue number 28106, Qiagen, Crawley, West Sussex, UK) designed to purify single or double stranded DNA fragments from PCR. 250 µl of Buffer P1 (Qiagen, Crawley, West Sussex, UK) was added to 50 µl of PCR reaction and mixed by vortexing, the DNA decanted into a QIAquick column (Qiagen, Crawley, West Sussex, UK) and centrifuged for 60 seconds at 13000 rpm using an IEC micromax centrifuge. The flow-through was discarded and 750 µl of Buffer PE (Qiagen, Crawley, West Sussex, UK) was added to the column, which was centrifuged for 60 seconds at 13000 rpm. The flow through was again discarded and the column was centrifuged for a further 60 seconds to ensure that residual ethanol from Buffer PE had been removed. The column was placed in a clean 1.5 ml centrifuge tube and 50 µl of deionised water added. The centrifuge was stood for 60 seconds at room temperature before centrifugation at 13000 rpm for 60 seconds. Eluted DNA was stored at -20°C prior to sequencing.

2.12. Cloning of PCR products.

PCR products less than 100 base pairs long are too small to be sequenced directly by the equipment available in our laboratory. These products need to be inserted into a vector prior to sequencing. The PCR products of exons 5 and 7 were each less than 100 base pairs long and were therefore cloned into bacterial plasmids prior to sequencing. Cloning was performed using the TA cloning kit (Catalogue number K2000-40, Invitrogen, Paisley, UK), which contains the pCR[®]2.1 vector (Figure 2.2) and the chemically competent *Escherichia coli* strain INVαF' which has

Figure 2.2. Map of pCR[®]2.1 vector (www.invitrogen.com). The sequence of the multiple cloning site is shown with a PCR product inserted by TA cloning.



a transformation efficiency of 1×10^8 cfu/ μ g DNA. The TA cloning kit uses the enzyme *Taq* polymerase to add a 3' deoxyadenosine residue to the 3' ends of PCR products. The pCR[®]2.1 vector contains a 3' deoxythymidine residue that allows the PCR product to efficiently ligate with the vector.

2.12.1. Ligation

1 μ l of fresh PCR product was added to 5 μ l sterile water, 1 μ l 10x ligation buffer, 2 μ l PCR vector (25 ng/ μ l) and 1 μ l of T4 DNA ligase. The ligation reaction was incubated overnight at 14°C and centrifuged for 60 seconds at 8000 rpm using an IEC MicroMax centrifuge (Thermo Scientific, Basingstoke, UK).

2.12.2. Transformation

Vials containing INV α F' cells, which had been stored at -20°C were thawed on ice and 2 μ l of each PCR reaction was added to separate vials and mixed with a pipette. Vials were incubated on ice for 30 minutes and heat-shocked in a 42°C water bath for exactly 30 seconds before placing the vials on ice for a further 2 minutes. SOC medium is a nutritionally rich bacterial growth medium. 250 μ l SOC medium (2% Tryptone, 0.5% yeast extract, 10 mM NaCl, 2.5 mM KCl, 10 mM MgCl₂, 10 mM MgSO₄, 20 mM glucose) was added to each vial before placing in a shaking incubator at 37°C at 225 rpm for exactly 1 hour. After incubation the vials were placed on ice prior to culture.

2.12.3. Culture of transformed bacteria

Luria-Bertani (LB) agar plates (2.5 g NaCl, 2.5 g Bactotryptone, 1.25 g yeast extract, 3.75 g agar, 250 ml deionised water) containing 50 μ g/ml ampicillin were

prepared and 20 µl 200mg/ml 5-bromo-4-chloro-3-indolyl-beta-D-galactopyranoside (X-Gal) added to each agar plate. X-Gal is a colourless substance which is split by beta-galactosidase to produce a substance which is a deep blue colour. The pCR[®]2.1 vector contains the lacZ gene which contains DNA coding the beta galactosidase protein. Bacterial colonies arising from bacteria where ligation of PCR products has not occurred appear blue as the beta galactosidase gene remains intact. However, where ligation within the DNA coding for the Beta-galactosidase protein occurs the activity of this enzyme is lost and resulting colonies will appear white.

20 µl and 100 µl of solution was removed from each vial containing transformed organisms, plated on separate agar plates and placed in a 37°C incubator for at least 16 hours. The plates were placed in a 4°C cold room for about 1 hour to allow for proper colour development. The largest 5 white colonies were selected from each sample and transferred to a further LB plate which was placed in an incubator for at least 16 hours. A sample of each colony was added to a PCR master mix containing appropriate primers and PCR performed (Chapter 2.10.2). Electrophoresis of the PCR products obtained was performed on 6% polyacrylamide gels (Chapter 2.11) and a band on the gel of correct molecular size confirmed the successful ligation of PCR products. Colonies containing PCR products were removed from the agar plate, placed into 5 ml LB broth (2.5 g NaCl, 2.5 g Bactotryptone, 1.25 g yeast extract, 250 ml deionised water) containing 50 µg/ml ampicillin, incubated at 37°C for at least 16 hours and DNA extraction from the resulting bacterial cell suspension performed.

2.12.4. Purification of cloned PCR products.

DNA extraction of plasmid DNA was carried out using QIAprep spin miniprep kit (Catalogue number 27106, Qiagen, Crawley, West Sussex, UK). The bacterial suspension was centrifuged for 10 minutes at 3000 rpm in a Beckman J-6B centrifuge (Beckman Coulter, High Wycombe, Buckinghamshire, UK). The supernatant was discarded, the pelleted bacterial cells resuspended in 250 µl Buffer P1 containing RNase (Qiagen, Crawley, West Sussex, UK) and transferred to a 1.5 ml eppendorff tube. 250 µl lysis Buffer P2 (Qiagen, Crawley, West Sussex, UK) was added, the eppendorff inverted 4 to 6 times, 350 µl Buffer N3 (Qiagen, Crawley, West Sussex, UK) for protein purification added to the cell suspension and the eppendorff inverted a further four to six times to mix thoroughly. Centrifugation was performed at 13000 rpm for 10 minutes using an Eppendorff 5415D centrifuge (Eppendorff, Hamburg, Germany). The supernatant obtained was transferred to a QIAprep column which was centrifuged for 60 seconds at 13000 rpm, the flow-through discarded and 500 µl Buffer PB added to the spin column which was centrifuged for a further 60 seconds to remove any trace nuclease activity. The flow-through was discarded and 750 µl Buffer PE containing ethanol added to the spin column which was centrifuged for a further 60 seconds. The flow-through was again discarded and the column centrifuged for an additional 60 seconds to remove any remaining buffer. The QIAprep column was placed in a clean 1.5 ml eppendorff tube and 50 µl of deionised water added. The tube was allowed to stand at room temperature for 60 seconds and then centrifuged for 60 seconds at 13000 rpm. Eluted DNA was stored at -20°C prior to sequencing.

2.13. DNA sequencing.

The amount of template used in the sequencing reaction was dependent on the size of the DNA fragment. The DNA concentration for each product was first calculated (Chapter 2.9) and a working concentration between 0-100 fmol was calculated according to the manufacturer's guidelines (Beckman Coulter, High Wycombe, Buckinghamshire, UK). The sequencing reactions were prepared in 0.2 ml tubes (Abgene, Epsom, UK). All reactions were kept on ice until required. PCR product (1-10 µl) and deionised water was added to each other to a total volume of 10 µl. The contents were mixed by vortexing and centrifuged at 13000 rpm for 30 seconds. To ensure separation of PCR products, the tubes were heated for 60 seconds at 96°C using a DNA engine thermal cycler (MJ Research PTC-2000 Peltier, MJ Research, Hemel Hempstead, Hertfordshire, UK) and cooled on ice. Specific primer (2.0 µl) at a concentration of 10 pmoles/µl was added to each tube followed by 8.0 µl quick start master mix (Beckman Coulter, High Wycombe, Buckinghamshire, UK). For each PCR product, samples were prepared with both forward and reverse primers separately in order to confirm the presence of any mutations, thus ensuring that any mutations found were not artefacts of the sequencing analysis. The prepared solutions were mixed by vortexing and centrifuged at 13000 rpm for 30 seconds prior to thermal cycling. Thermal cycling proceeded at 96°C for 20 seconds, 50°C for 20 seconds and 60°C for 4 minutes for 30 cycles followed by ethanol precipitation. DNA sequence analysis was performed using a Beckman Coulter CEQ 2000 DNA analysis system (Beckman Coulter, High Wycombe, Buckinghamshire, UK). Sequencing data was compared with the known wild-type sequence of the p53 gene to characterise mutations identified by RSM assay.

3. Results.

3.1. Results from fluorescence *in-situ* hybridisation investigations.

Fluorescence *in-situ* hybridisation (FISH) was successfully performed on test samples (near to the enterovesical anastomosis) prepared from all fifteen patients (Table 3.1) and on twelve of the fifteen control specimens of the native bladder (Table 3.2). In three patients, FISH on the control sample was unsuccessful because of too much background signal due to excessive debris on the slides (one patient), unsuccessful hybridisation (one patient) and an inadequate touch sample preparation with insufficient cells (one patient).

Normal values were calculated using the results obtained from the control specimens (Tables 3.3 and 3.4). The cut-off criteria used in categorising results as normal versus abnormal were defined as three standard deviations from the mean of the control values and calculated for both monosomy and trisomy for each of the chromosomes studied. The use of such a threshold is consistent with the analysis performed by other researchers (Huang *et al*, 1999; Botti *et al*, 2000; Debiec-Rychter *et al*, 2001). Chromosomal counts exceeding the normal range were considered abnormal. Normal ranges were used to determine which patients had excess numbers of aneuploid cells and the numbers of patients whose biopsies were abnormal (Table 3.5).

No abnormal findings were found in any of the control samples (Figure 3.1). Significant aneuploidy was detected in tissue obtained from near to the enterovesical

Table 3.1. Number of chromosomes per cell for two hundred cells scored from specimens taken from the enterovesical anastomosis (abnormal findings are highlighted in red).

Patient No.	Age /years	Time between operation and biopsy /years	Chromosome 8			Chromosome 9			Chromosome 18		
			≤1	2	≥3	≤1	2	≥3	≤1	2	≥3
1	28	12	10	189	0	7	193	0	12	188	0
2	39	10	6	194	0	24	175	1	3	197	0
3	21	6	7	192	1	53	147	0	18	181	1
4	62	16	6	194	0	32	167	1	37	162	1
5	23	11	8	191	1	6	194	0	30	169	1
6	61	12	6	194	0	7	193	0	41	158	1
7	29	11	6	193	1	7	193	0	24	174	2
8	34	15	23	177	0	4	196	0	21	171	8
9	31	13	7	192	1	17	181	2	4	168	28
10	25	14	5	195	0	15	184	1	6	189	5
11	48	17	5	194	1	28	171	1	3	164	32
12	29	13	10	188	1	35	177	2	8	188	4
13	38	18	8	185	7	5	194	1	29	177	4
14	43	11	3	197	0	44	155	2	7	190	3
15	27	9	3	197	0	21	177	2	2	176	22

Table 3.2. Number of chromosomes per cell for two hundred cells scored from specimens taken from the native bladder remnant (control biopsies).

Patient No.	Chromosome 8			Chromosome 9			Chromosome 18		
	≤1	2	≥3	≤1	2	≥3	≤1	2	≥3
1	7	192	1	6	194	0	7	193	0
2	7	193	0	7	193	0	4	196	0
3	8	191	1	6	194	0	7	192	1
4	5	194	1	7	193	0	5	193	2
5	5	194	1	5	195	0	7	193	0
6	Signal to noise ratio poor due to excess background debris on slide								
7	Unsuccessful hybridisation								
8	4	196	0	6	194	0	5	194	1
9	3	197	0	5	195	0	4	193	3
10	3	196	1	6	194	0	3	195	2
11	7	193	0	5	194	1	8	191	1
12	4	196	0	6	193	1	3	195	2
13	Too few cells on touch sample preparations								
14	4	196	0	9	189	2	4	195	1
15	3	197	0	4	196	0	1	197	2

Table 3.3. Frequency of aneuploidy per two hundred cells in control samples. Normal values are those within 3 standard deviations from the mean (extent of vertical line) and the median values are shown as a horizontal line within the vertical line.

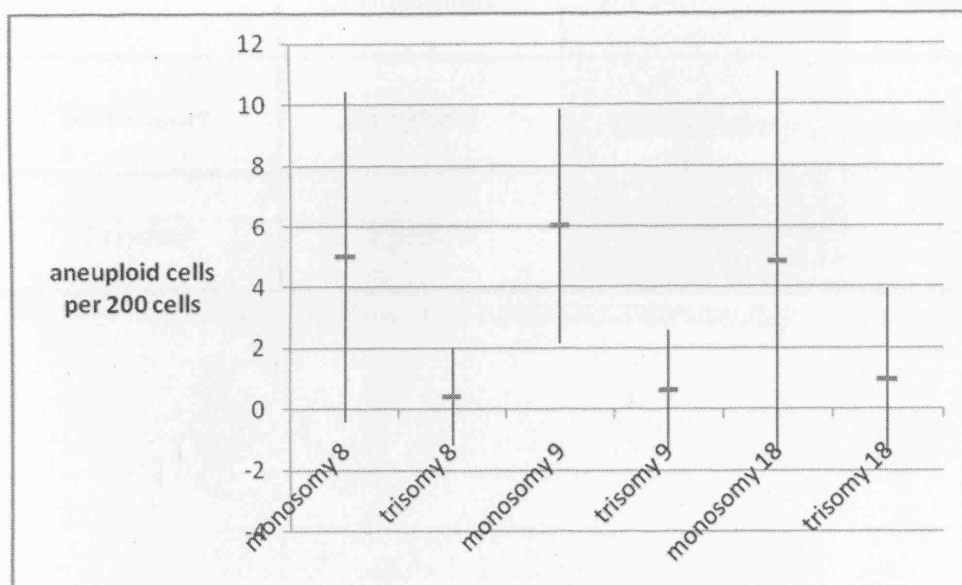


Table 3.4. Normal values for frequency of aneuploidy per two hundred cells, derived from the means and standard deviations of the control values.

	Chromosome 8	Chromosome 9	Chromosome 18
Monosomy	<11 cells	<10 cells	<12 cells
Trisomy	<3 cells	<3 cells	<4 cells

Table 3.5. Numbers of patients with abnormally high incidence of aneuploid cells at the anastomosis (n = 15).

	Chromosome 8	Chromosome 9	Chromosome 18
Monosomy	1 patient	9 patients	8 patients
Trisomy	1 patient	0 patients	7 patients

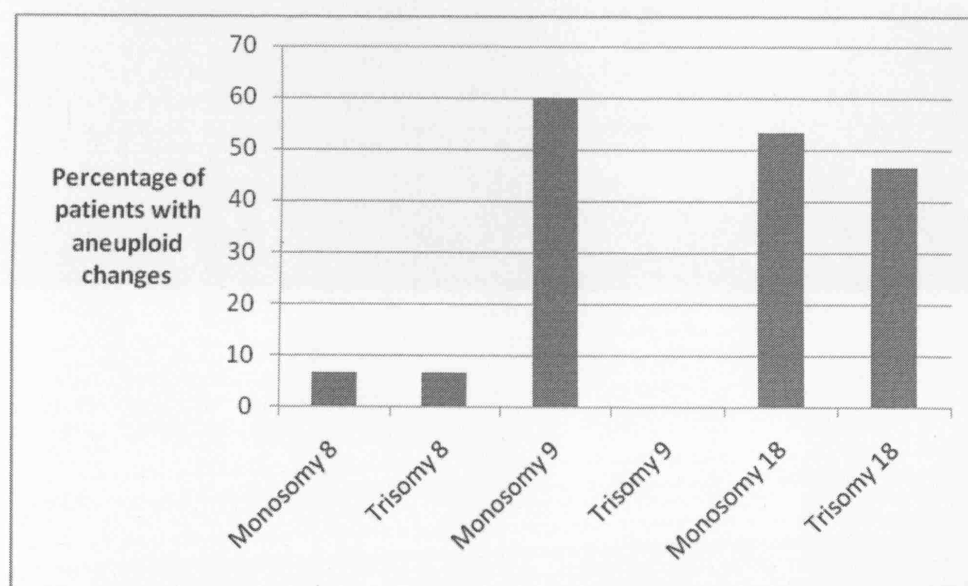
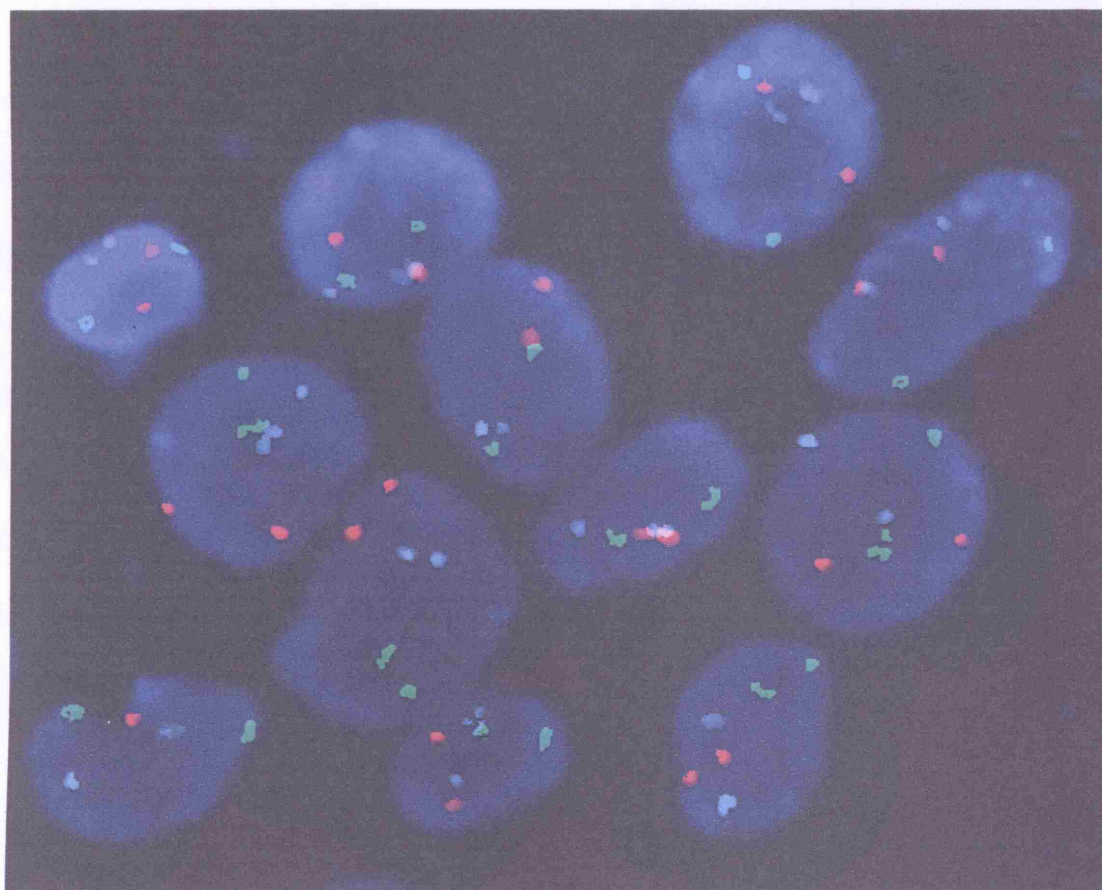


Figure 3.1. Fluorescence *in-situ* hybridisation of diploid cells obtained from a control sample. Chromosomes 8, 9 and 18 are visualised with red, green and aqua probes respectively.



anastomosis of all fifteen patients. Alterations in chromosome 18 copy number were the most frequent abnormal findings and were almost evenly divided between monosomy (Figure 3.2) and trisomy (Figure 3.3). Two patients exhibited both a gain and loss of chromosome 18. Nine patients were monozygous for chromosome 9 (Figure 3.4) but no cases of trisomy 9 were found. Only two patients had abnormalities of chromosome 8 copy number; monosomy 8 and trisomy 8 were each found in one patient. It is of interest that in both patients with abnormalities of chromosome 8, monosomy and trisomy 18 were also present. Since several different types of chromosomal instability were identified in these two patients it is possible that they are at an increased risk of malignant transformation. Neither the total number of aneuploid cells found, nor the extent of aneuploidy of any particular chromosome, was influenced by the latent period between the time of constructing a clam ileocystoplasty and the collection of biopsies (Figures 3.5 to 3.8). In addition, the age of the patient at the time of biopsy did not affect the number of aneuploid cells detected (Figure 3.9).

One biopsy from each patient was sent for histology. In all cases no dysplastic or malignant changes were reported although a few patients had mild inflammatory changes.

Figure 3.2. Fluorescence in-situ hybridisation of tissue obtained from a clam ileocystoplasty. Chromosomes 8, 9 and 18 are visualised with red, green and aqua probes respectively. Monosomy of chromosome 18 is clearly visualised (arrowed).

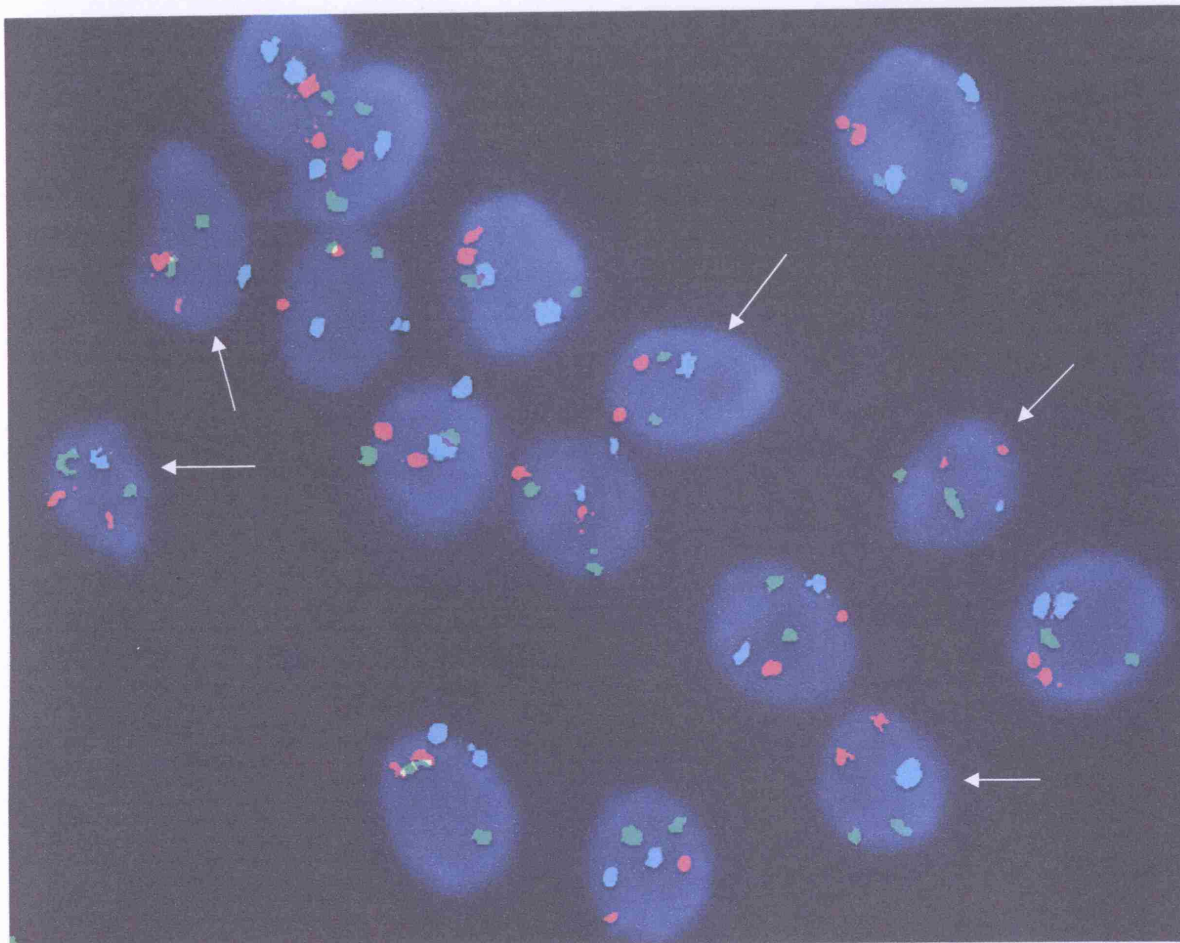


Figure 3.3. Fluorescence in-situ hybridisation of tissue obtained from a clam ileocystoplasty. Chromosomes 8, 9 and 18 are visualised with red, green and aqua probes respectively. Trisomy of chromosome 18 is clearly visible (arrowed).

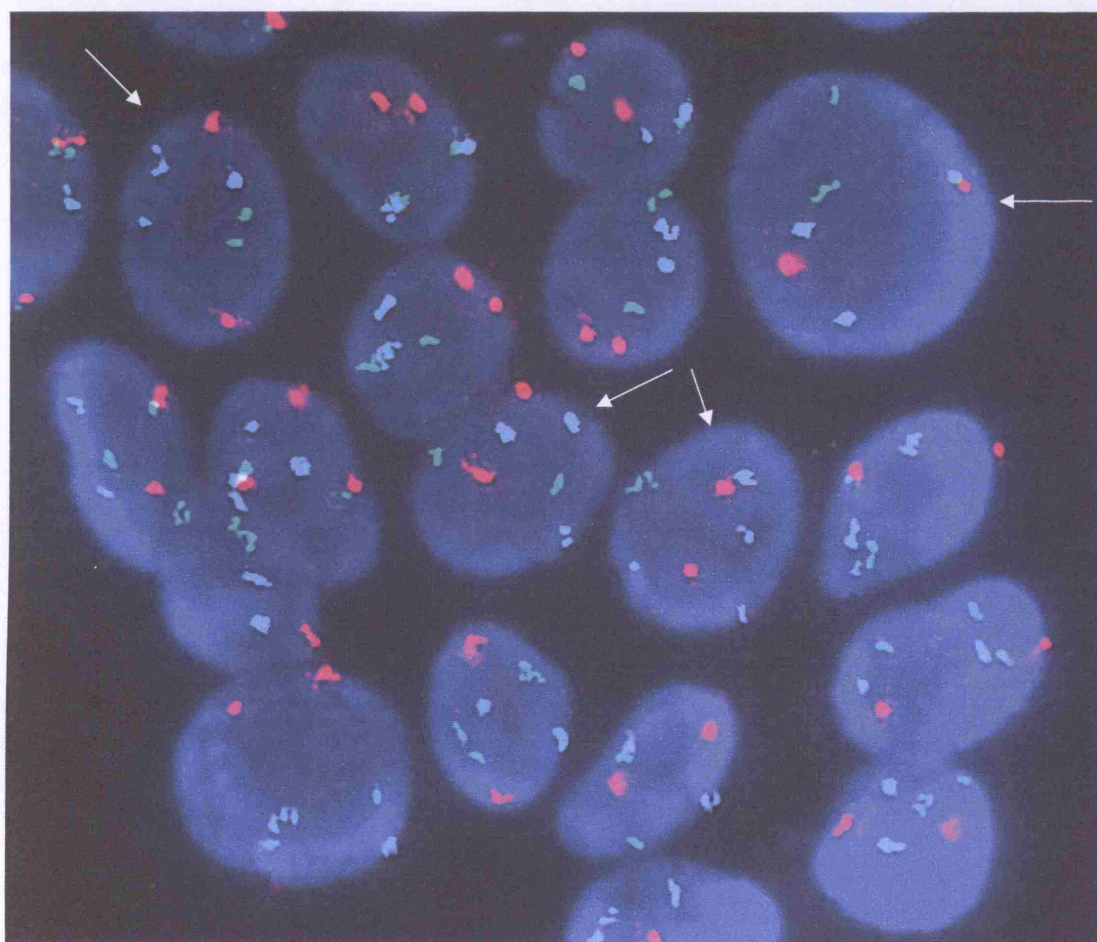


Figure 3.4. Fluorescence *in-situ* hybridisation of tissue obtained from a clam ileocystoplasty. Chromosomes 8, 9 and 18 are visualised with red, green and aqua probes respectively. Monosomy of chromosome 9 is clearly visualised (arrowed) in two of the cells.

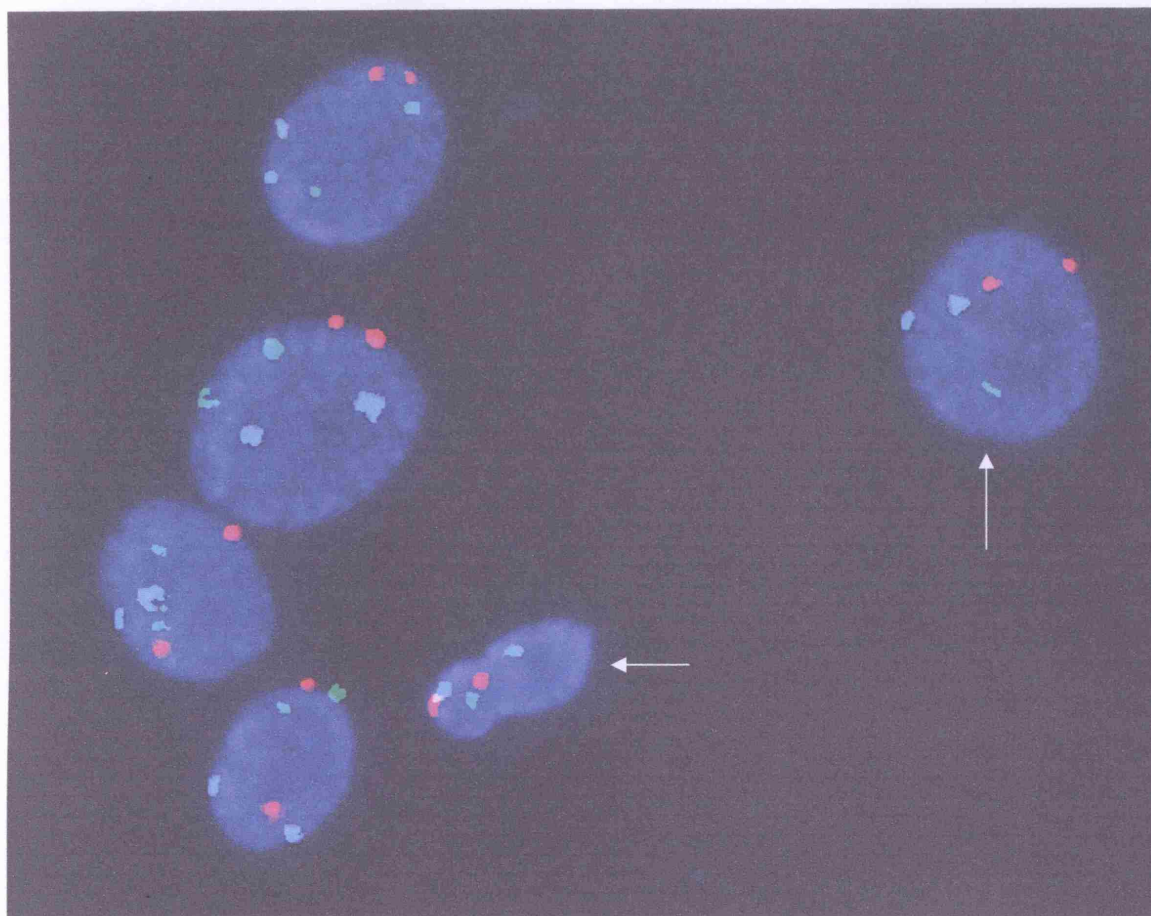


Figure 3.5. Regression analysis of the relationship between the combined number of aneuploid changes of chromosomes 8, 9 and 18 and time since operation.

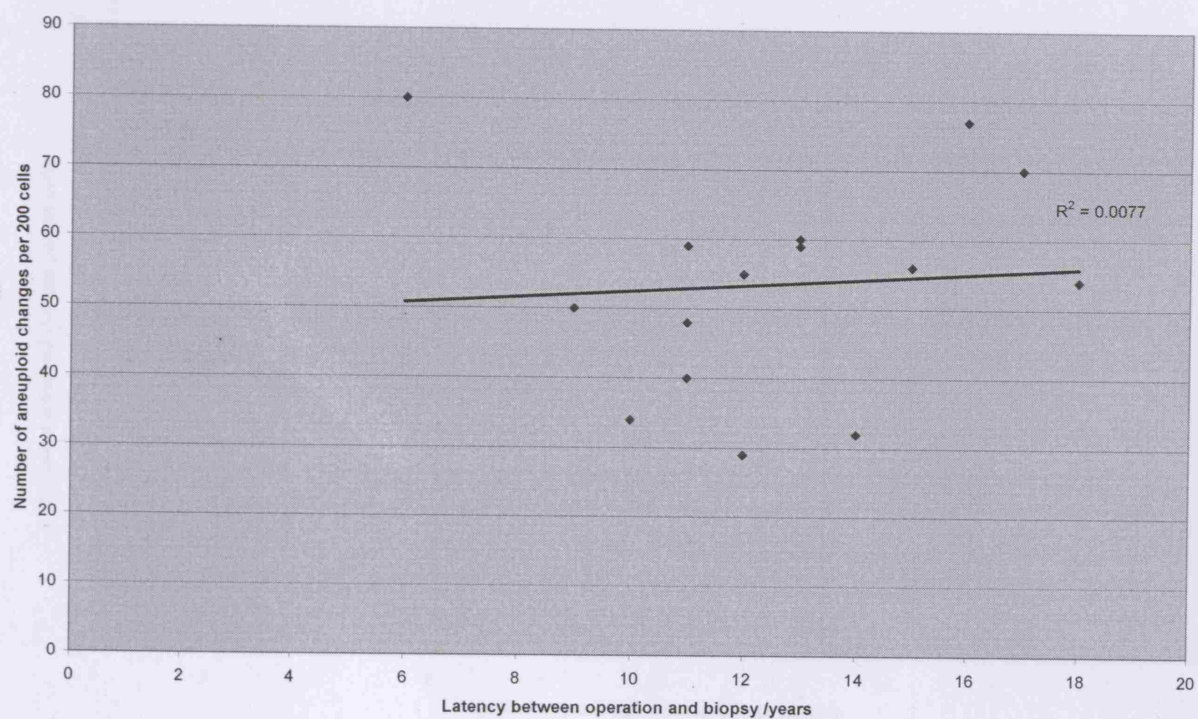


Figure 3.6. Regression analysis of the relationship between the number of aneuploid changes of chromosome 8 and time since operation.

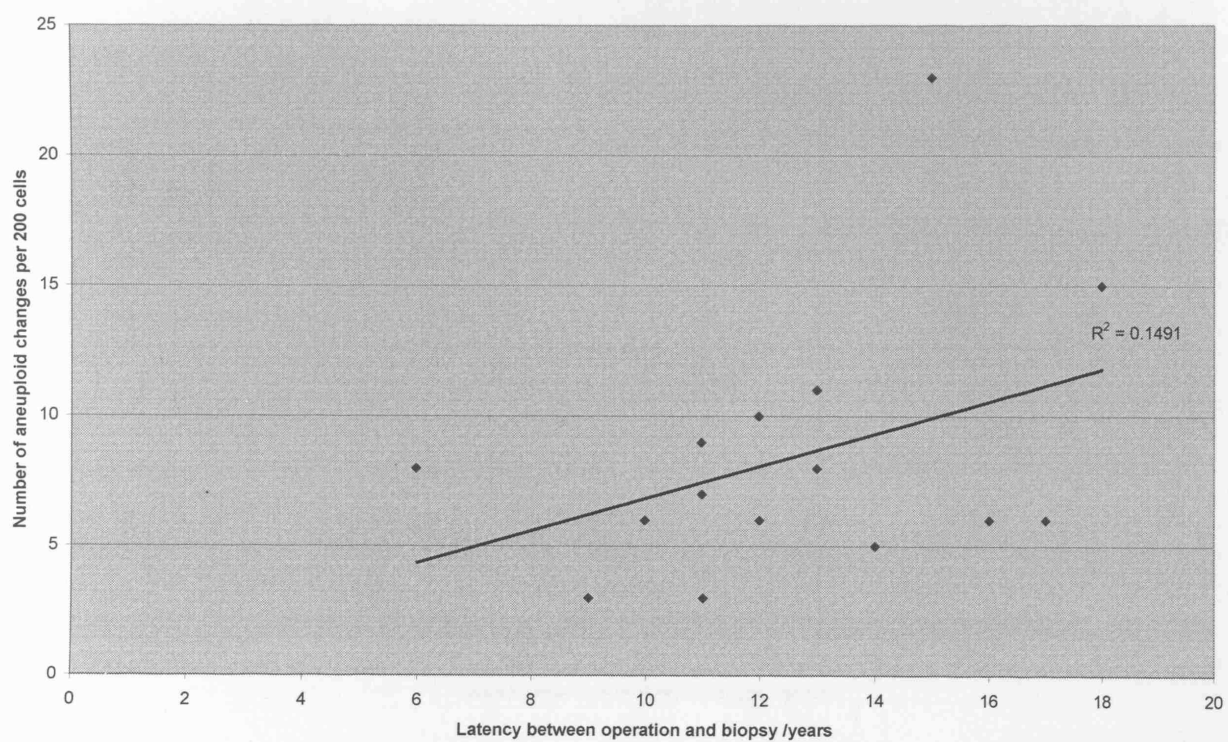


Figure 3.7. Regression analysis of the relationship between the number of aneuploid changes of chromosome 9 and time since operation.

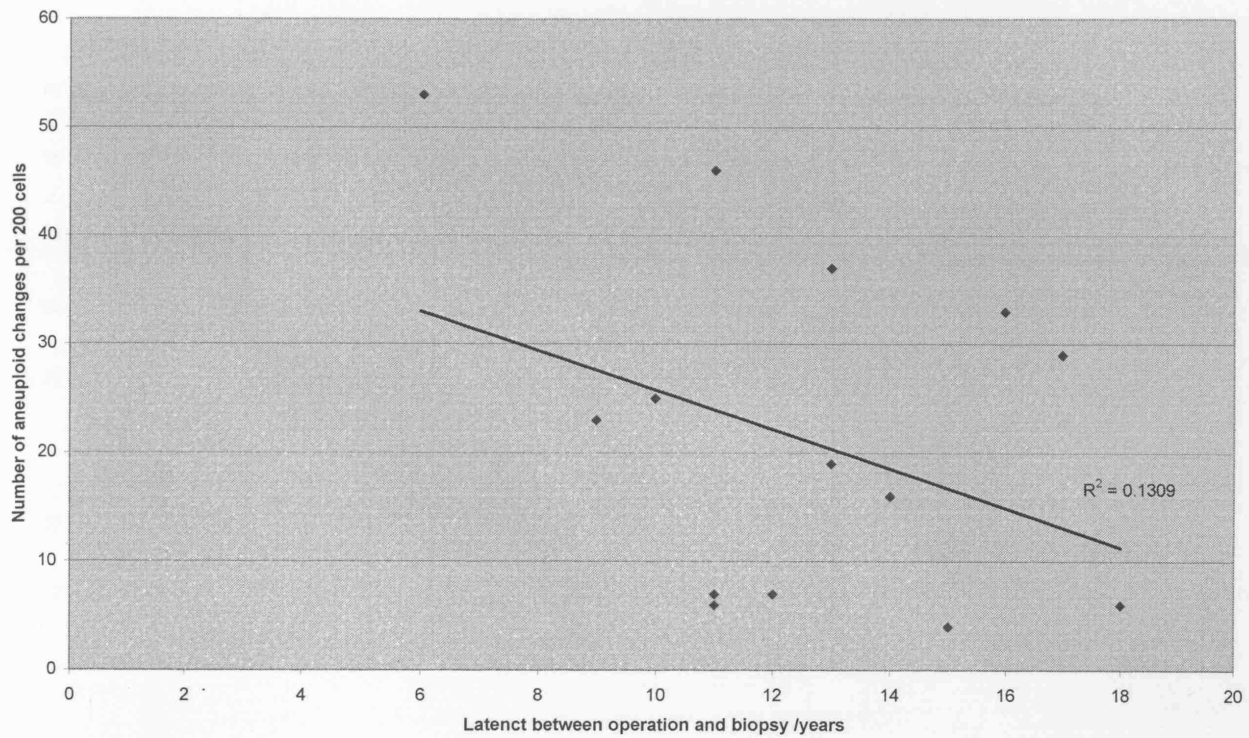


Figure 3.8. Regression analysis of the relationship between the number of aneuploid changes of chromosome 18 and time since operation.

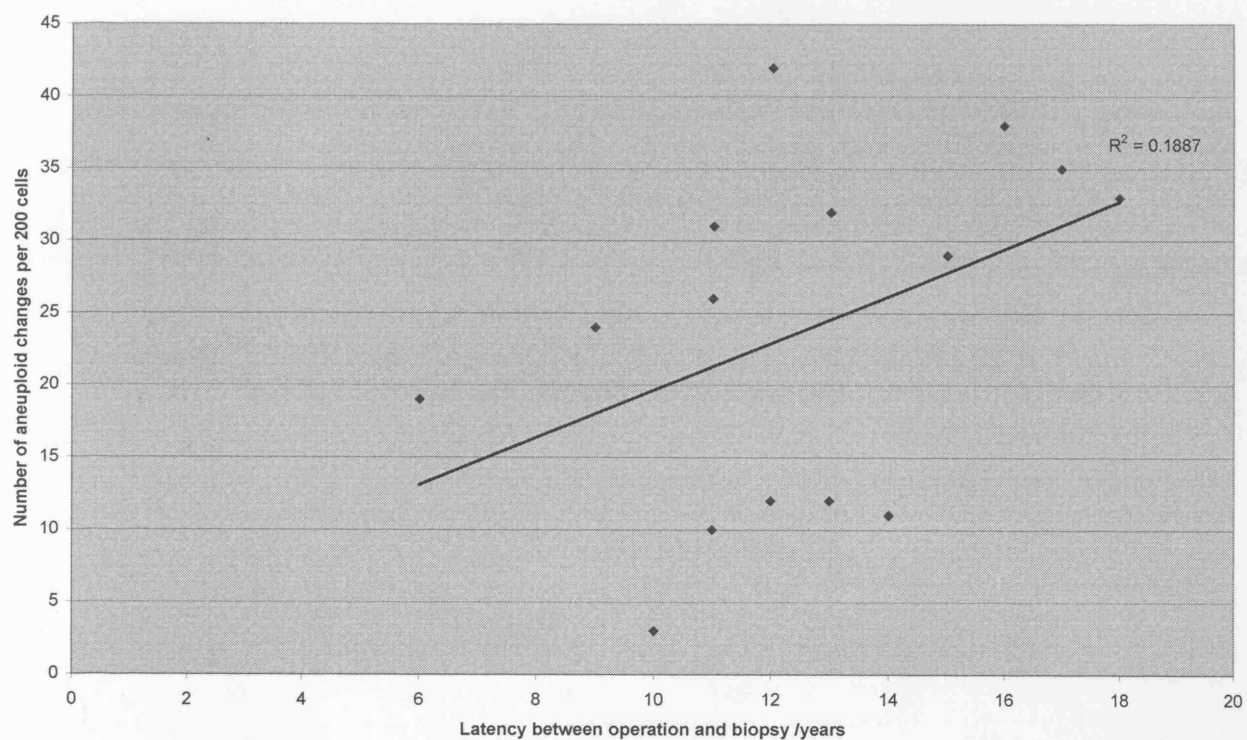
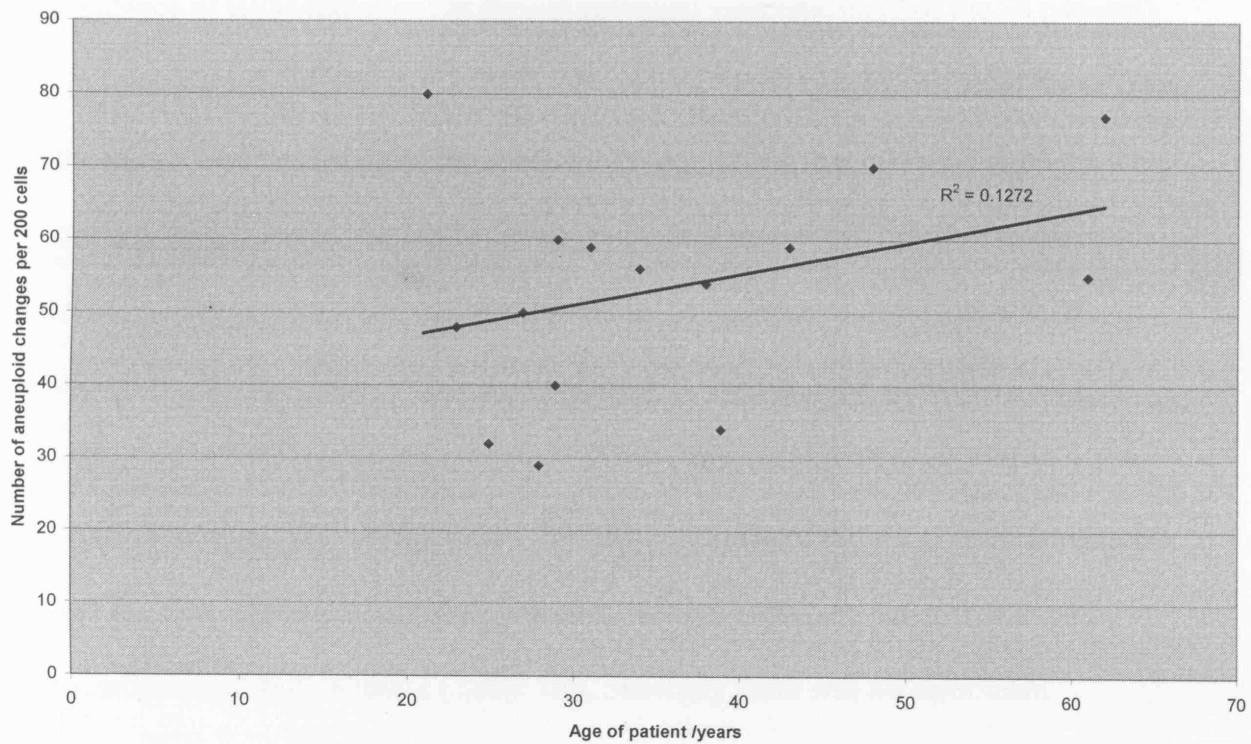


Figure 3.9. Regression analysis of the relationship between the combined number of aneuploid changes of chromosomes 8, 9 and 18 and age of patients.

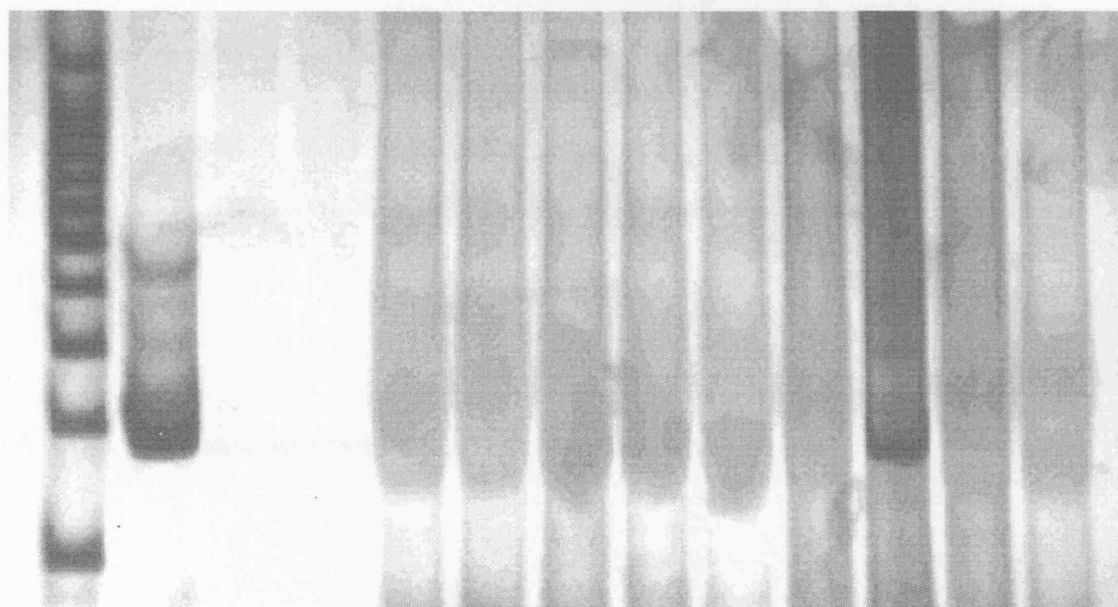


3.2. Results from Restriction Site Mutation assay investigations.

The Restriction Site Mutation (RSM) assay was performed on DNA extracted from biopsies taken from the native bladder remnant (controls) in thirty-seven of the thirty-eight patients. In one patient control biopsies had not been collected. No p53 mutations were detectable at the five hotspots in any of the control biopsies. The incidence of RSM mutations at the enterovesical anastomosis (7 of the 38 patients) was significantly higher ($p = 0.0057$ Fishers Exact test) than in urothelial tissue from the native bladder distant to the anastomosis suggesting that mutation artefacts were not introduced during the methodology and that all mutations detected in this study were genuinely present in anastomotic tissue. Undigested mutated DNA PCR products were identified on polyacrylamide gels post-stained with silver (Figure 3.10) and the mutations characterised by DNA sequencing (Figures 3.11 to 3.13). Age at operation, age at biopsy and the latent period between the time of operation and the time of study was not significantly different between patients with p53 mutations and those without (Table 3.6). Similarly there was no significant difference in the incidence of p53 mutations at the enterovesical anastomosis between males and females or between patients who underwent a clam enterocystoplasty for a neuropathic bladder and those with a non-neuropathic bladder (Table 3.7).

A mutation was identified in three of the sixteen male patients (18.7 percent) and four of the twenty-two female patients (18.2 percent). The mean age of patients who had a mutation identified in an anastomotic bladder biopsy was 34.9 years (median 28 years). The mean age of patients where no mutation was identified was 35.9 (median 31 years). In patients where a mutation was identified the mean latency

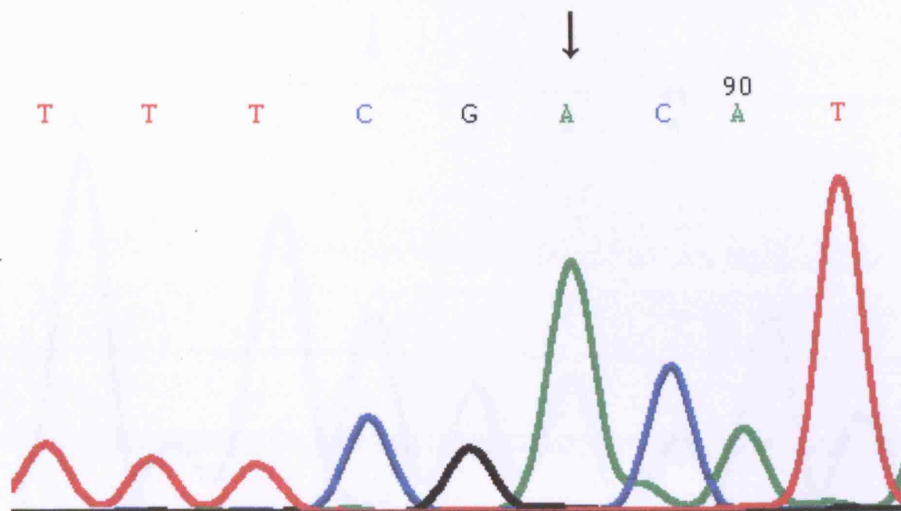
Figure 3.10. Polyacrylamide gel identifying a p53 mutation at codon 213 by RSM analysis using *Taq I*. A 100 base pair DNA ladder, positive and negative control are shown on the left of the gel.



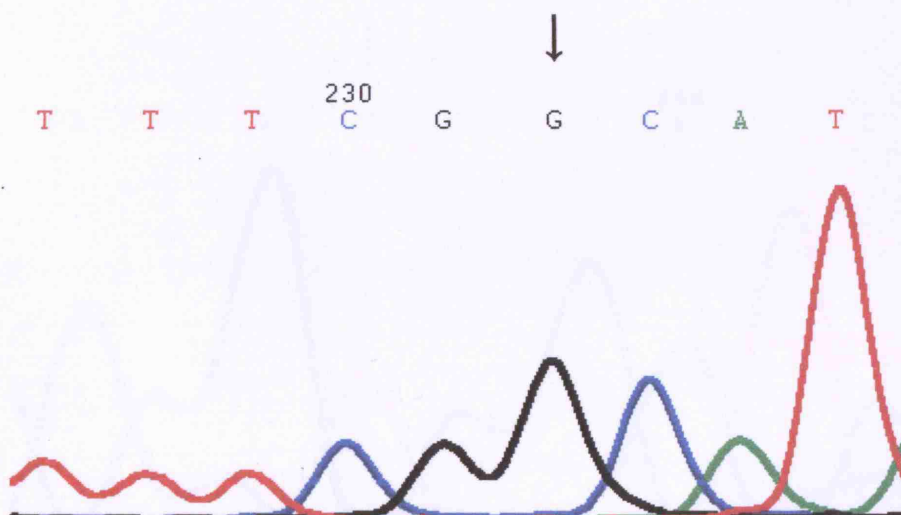
↑
188 base pair PCR product
identifying a p53 mutation

Figure 3.11. Sequencing data demonstrating CGA→CGG mutation at codon 213 at a *Taq* I restriction site.

Wild-type DNA.



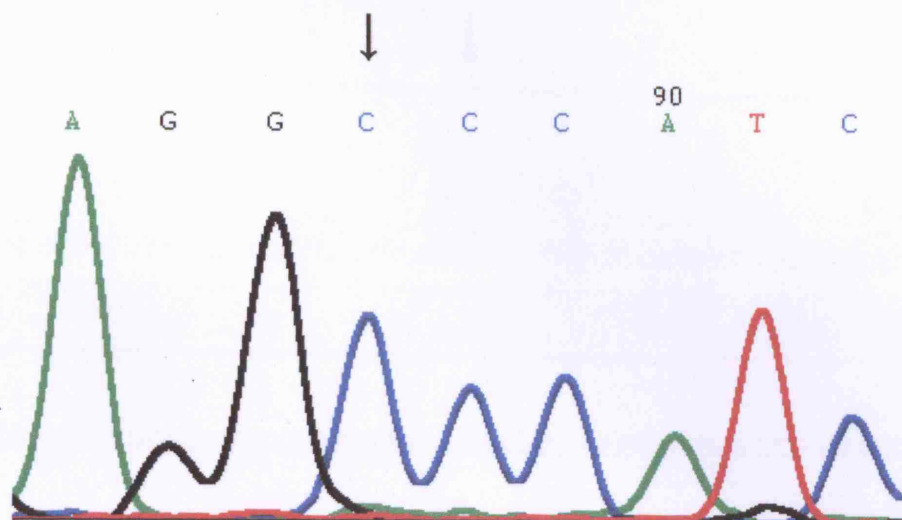
DNA from the anastomosis of a clam ileocystoplasty.



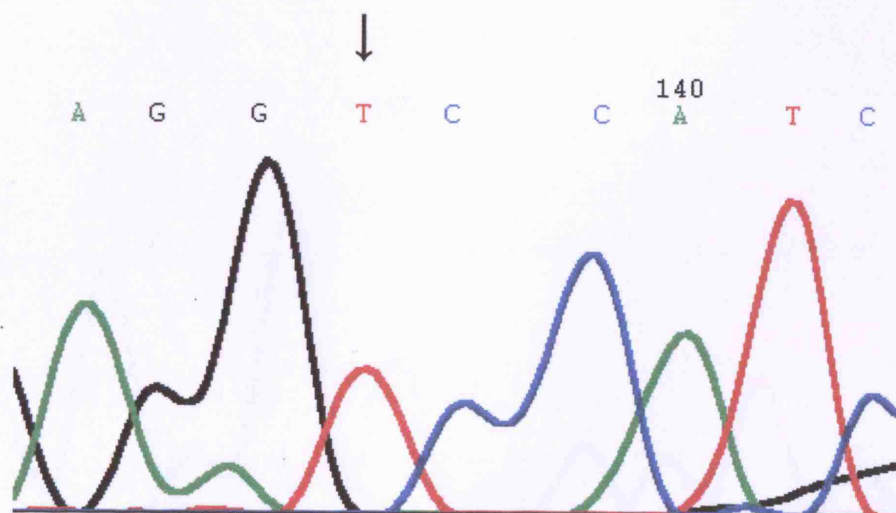
DNA is constructed from a combination of the four nucleotides adenine (A), cytosine (C), guanine (G) and thymine (T). DNA sequencing determines the sequence of nucleotides in a length of DNA. The sequencer detects signals from nucleotides labelled with four different fluorescent dyes. These signals are then plotted on a sequence trace. A DNA trace obtained from the anastomosis of a clam enterocystoplasty is shown. The mutated nucleotide is highlighted with an arrow. A sequence trace from wild-type (normal) DNA is also shown for comparison.

Figure 3.12. Sequencing data demonstrating CCC→TCC mutation at codon 250 at a *Hae* III restriction site.

Wild-type DNA.



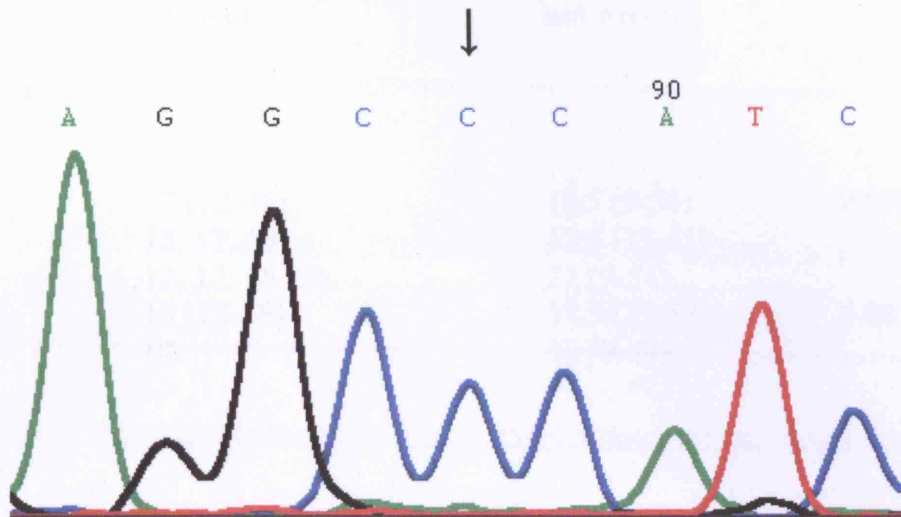
DNA from the anastomosis of a clam ileocystoplasty.



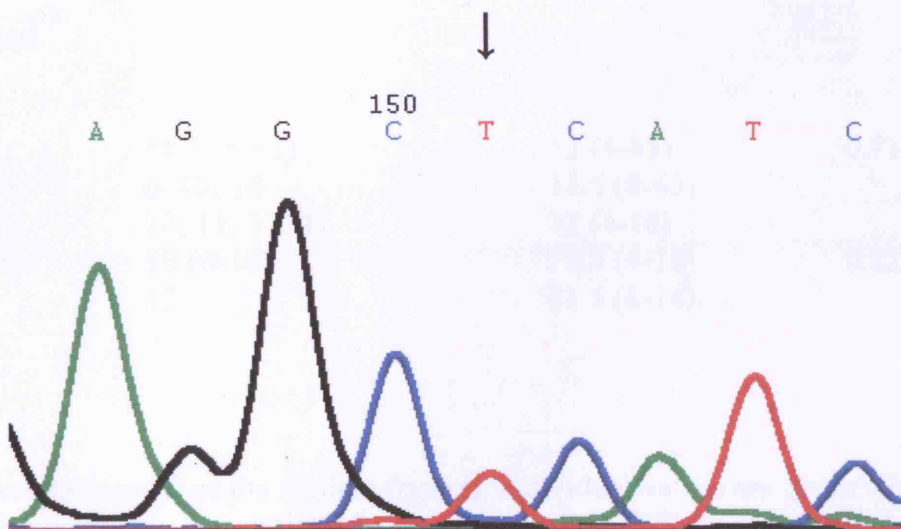
DNA is constructed from a combination of the four nucleotides adenine (A), cytosine (C), guanine (G) and thymine (T). DNA sequencing determines the sequence of nucleotides in a length of DNA. The sequencer detects signals from nucleotides labelled with four different fluorescent dyes. These signals are then plotted on a sequence trace. A DNA trace obtained from the anastomosis of a clam enterocystoplasty is shown. The mutated nucleotide is highlighted with an arrow. A sequence trace from wild-type (normal) DNA is also shown for comparison.

Figure 3.13. Example of sequencing data demonstrating a CCC→CTC mutation at codon 250 at a *Hae* III restriction site.

Wild-type DNA.



DNA from the anastomosis of a clam ileocystoplasty.



DNA is constructed from a combination of the four nucleotides adenine (A), cytosine (C), guanine (G) and thymine (T). DNA sequencing determines the sequence of nucleotides in a length of DNA. The sequencer detects signals from nucleotides labelled with four different fluorescent dyes. These signals are then plotted on a sequence trace. A DNA trace obtained from the anastomosis of a clam enterocystoplasty is shown. The mutated nucleotide is highlighted with an arrow. A sequence trace from wild-type (normal) DNA is also shown for comparison.

Table 3.6. Demographic details of patients with or without p53 mutations at the enterovesical anastomosis following an enterocystoplasty.

	P53 mutations present	p53 mutations not present	p value
Age at operation (years)			
All patients	17 (12-48)	18.5 (9-54)	0.658
Males	15, 17, 48	17.5 (11-41)	
Females	12, 12, 18, 20	22 (9-54)	0.881
Neuropathic	15 (12-48)	17.5 (12-54)	
Lifelong	18	18 (9-49)	
Age at biopsy (years)			
All patients	28 (21-61)	33 (18-65)	0.634
Males	21, 28, 58	28 (22-63)	
Females	22, 23, 31, 61	35 (18-65)	0.688
Neuropathic	23 (21-58)	28.5 (22-65)	
Lifelong	31	25 (18-61)	
Latent period (months)			
All patients	11.5 (6-41)	12 (4-43)	0.713
Males	6, 10, 14	11.5 (8-43)	
Females	10, 11, 13, 41	12 (4-18)	0.821
Neuropathic	10 (6-16)	10.5 (4-18)	
Lifelong	13	11.5 (8-14)	

The results are expressed as the median (range). Individual values are given when numbers are less than 6. The statistical significance of the differences between the groups was evaluated by the Mann Whitney U test. No formal comparisons were made when numbers there was less than five patients.

Table 3.7. Incidence of p53 mutations at the enterovesical anastomosis according to gender and the indication for the construction of an enterocystoplasty.

	P53 mutations present	p53 mutations not present	p value
Males	3 (17.6%)	14	0.6546*
Females	4 (19.0%)	17	
Neuropathic	5 (23.8%)	16	0.6463**
Lifelong	1 (10%)	9	
Idiopathic	0	4	
Tubercular cystitis	1	1	
Post radiotherapy	0	1	

Incidence of p53 mutations at the enterovesical anastomosis in *males versus females and **lifelong versus neuropathic patients (Fisher's Exact Test).

between the time of operation and biopsy was 14.6 years (median 11 years). In patients where no mutation was identified the mean latency between the time of operation and biopsy was 11.9 years (median 12 years). Logistic regression analysis indicated that age-related variables, gender or the indication for clam ileocystoplasty did not independently influence the risk of p53 mutations at the enterovesical anastomosis (Table 3.8).

Table 3.9 contains the details of all the p53 mutations detected at the enterovesical anastomosis in patients who had undergone a clam ileocystoplasty. Seven of the thirty-eight patients (18.4 percent) had a mutation in one of their biopsies. One patient had a mutation at codon 213, three patients had mutations of codon 248 and three patients had mutations of codon 250. Therefore, a total of seven mutational events were detectable in the one hundred and ninety RSM experiments performed. No mutational events were identified from a further one hundred and eighty-five RSM experiments on control DNA.

It is noteworthy that six of the seven mutations highlighted by this study were exon 7 mutations in codons 248 and 250 suggesting that mutations were preferentially induced in this region. The restriction site for the endonuclease *Hae* III covers both codons 249 and 250 and was chosen primarily to investigate changes at codon 249 of the p53 tumour suppressor gene which is a recognised hotspot for mutations. It is of interest that mutations of codon 250 have been only occasionally reported (p53 mutation database, www.iarc.fr) but in this study three of the seven mutations were found at codon 250.

Table 3.8. Logistic regression analysis of demographic variables on the incidence of p53 mutations at the enterovesical anastomosis following an enterocystoplasty.

	Wald statistic	$G\chi^2$ (likelihood ratio)	Significance
Age at operation	0.376	1.625	0.540
Age at biopsy	0.423	0.589	0.515
Latent period between time of operation and biopsy	0.445	1.732	0.505
Gender	0.000	1.007	0.994
Indication for operation	0.117	1.252	0.732

Table 3.9. P53 mutations identified by RSM analysis in patients who have undergone clam ileocystoplasty.

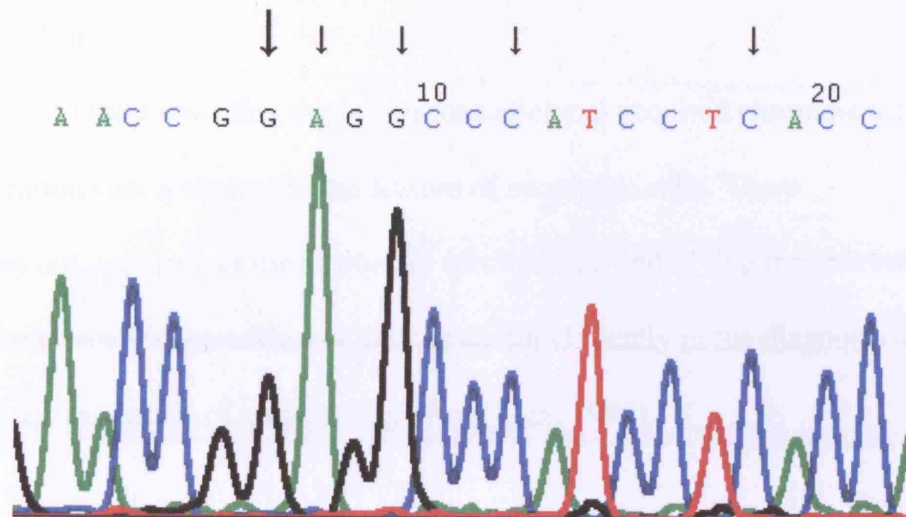
Exon	Codon	Mutation	Amino-acid change
6	213	CGA→CGG	Silent mutation (arginine)
7	248	CGG→CGC (within complex mutation)	Silent mutation (arginine)
7	248	CGG→TGG	Arginine→tryptophan
7	248	Unable to sequence RSM product	
7	250	CCC→TCC	Proline→serine
7	250	CCC→CTC	Proline→leucine
7	250	CCC→TCC	Proline→serine

Six of the seven mutations were characterised by sequencing. In one case it was not possible to characterise the mutation by sequencing the PCR product due to technical difficulties because of the poor quality of the PCR product. Of the six mutations characterised by sequencing, four (sixty-seven percent) were GC → AT transitions, three of them at CpG sites. Five of the six mutations characterised by sequencing only contained one point mutation. However, a CGG → CGC transversion identified at codon 248 was part of a complex mutation where several point mutations were identified by sequencing exon 7 of the p53 gene (Figure 3.14). Normal control samples had been obtained for all affected patients.

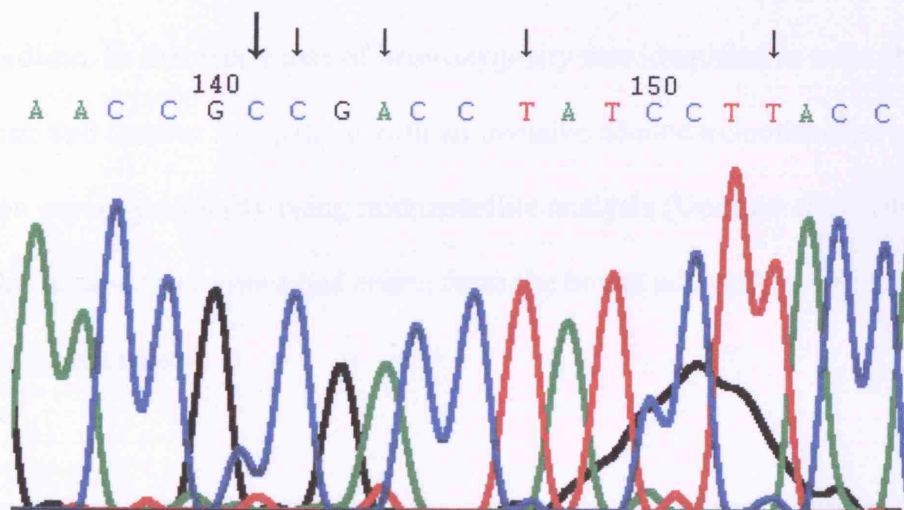
The 38 patients used for RSM analysis included the cohort of 15 patients used for FISH analysis. However, no p53 mutations were identified by RSM analysis in any of the 15 patients studied using FISH.

Figure 3.14. Sequencing data demonstrating complex mutation including CGG→CGC mutation at codon 248 at *Msp* I restriction site.

Wild-type DNA.



DNA from the anastomosis of a clam ileocystoplasty.



DNA is constructed from a combination of the four nucleotides adenine (A), cytosine (C), guanine (G) and thymine (T). DNA sequencing determines the sequence of nucleotides in a length of DNA. The sequencer detects signals from nucleotides labelled with four different fluorescent dyes. These signals are then plotted on a sequence trace. A DNA trace obtained from the anastomosis of a clam enterocystoplasty is shown. The mutated nucleotides are highlighted with arrows. A sequence trace from wild-type (normal) DNA is also shown for comparison.

4. Genetic Analysis of a Clam Cancer.

4.1. Introduction.

It is well established that the occurrence of clonal acquired chromosomal or genetic aberrations are a characteristic feature of neoplastic cells. These abnormalities not only reflect the molecular mechanisms underlying the process of tumour initiation and progression but may be useful clinically in the diagnosis and in determining the prognosis of cancers (Sandberg *et al*, 1996).

The frequency of particular chromosomal and genetic changes identified on different cancers is very variable. The genetic changes leading to carcinoma formation within enterocystoplasty are not well understood (Chapter 1.4). Only one report on the genetic analysis of a tumour developing within an enterocystoplasty is listed on Medline. In that study loss of heterozygosity was identified in cells obtained from the urine and tumour in a patient with an invasive adenocarcinoma after augmentation caecocystoplasty using microsatellite analysis (Docimo *et al*, 1999). In this particular patient the tumour had arisen from the bowel adjacent to the enterovesical anastomosis.

Since there is a paucity of data on the genetic alterations that occur in tumours arising within an augmentation cystoplasty, the purpose of this part of the study was to identify chromosomal changes and mutations of the p53 gene in a clam cancer that may be associated with progression, invasion and metastasis.

4.2. Case report.

A fifty-three year old female presented with painless haematuria. She had spina bifida occulta and had undergone a clam ileocystoplasty at the age of forty-five for the treatment of a neuropathic bladder. A cystoscopy diagnosed a tumour of the anterior bladder remnant close to the enterovesical anastomosis. The patient underwent a laparotomy where it was found that the tumour involved the symphysis pubis. A radical cystectomy, hysterectomy, bilateral oophorectomy and ileal conduit were performed. Despite radiotherapy the patient died the following year.

Macroscopic examination revealed a 30mm by 20mm ulcerating tumour on the anterior wall of the bladder. The neoplasm was located in a urothelial area extending to within 10mm of the ileal margin. Microscopy revealed a moderately differentiated squamous cell carcinoma invading into the perivesical fat. The urothelium adjacent to the tumour displayed features of carcinoma *in-situ*, these changes extending as far as, but not crossing the ileovesical anastomosis. The bladder and bowel epithelium distant from the tumour were inflamed with no evidence of dysplasia. Material taken from the symphysis pubis showed invasive carcinoma in the fibrous tissue but no involvement of bone.

4.3. Methods.

4.3.1. Fluorescence *in-situ* hybridisation on touch preparations.

Part of the tumour was dissected from the cystectomy sample by Dr. D. Griffiths, consultant histopathologist. Touch sample preparations were prepared from the fresh tumour material (Chapter 2.4) and stored at -20°C. Pepsin digestion of the cells was performed (Chapter 2.5) prior to fluorescence *in-situ* hybridisation (Chapter 2.6). Orange, green and aqua fluorescent centromeric probes (Vysis) were used for chromosomes 8, 9 and 18 respectively. The slides were visualised under fluorescence microscopy and the signals of two hundred cells scored (Chapter 2.6.5). The findings were compared with those obtained from the normal controls (Table 3.2).

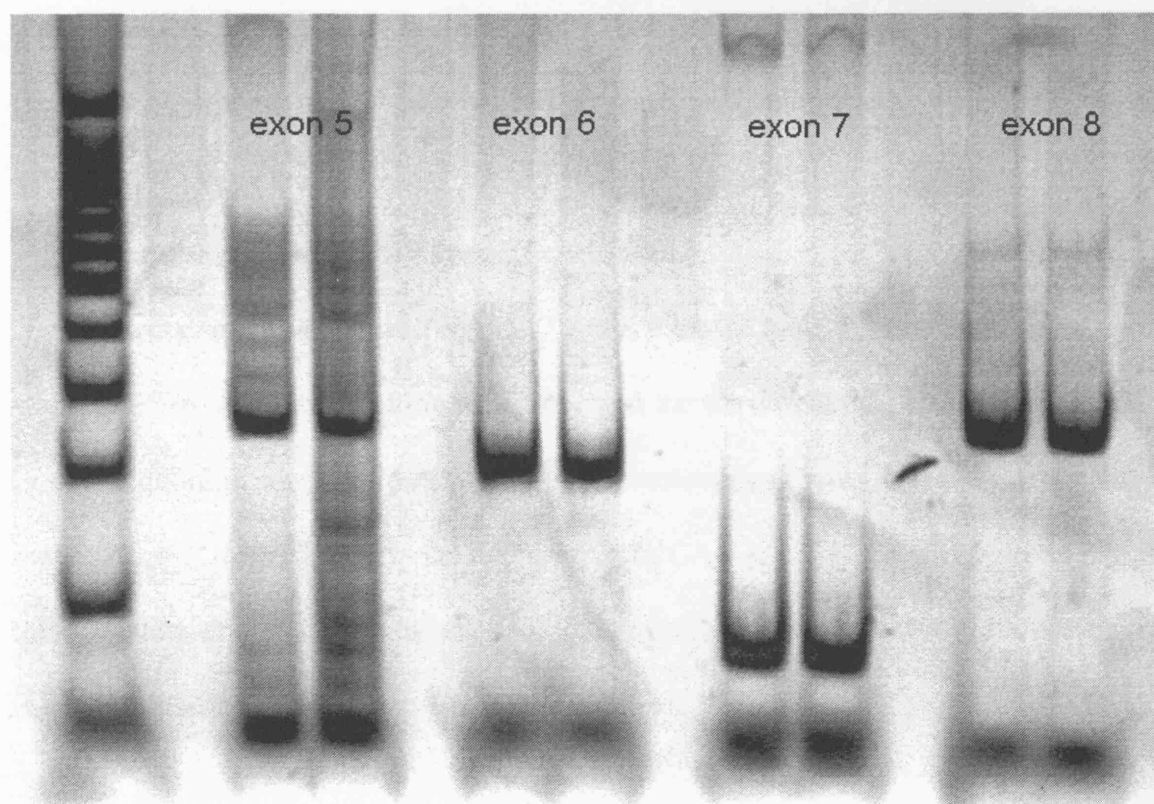
4.3.2. Sequencing the p53 tumour suppressor gene.

DNA was extracted from the tumour specimen (Chapter 2.8). The DNA concentration was adjusted to 100ng/μl (Chapter 2.9) and stored at -20°C. PCR products were obtained for exons five to eight of the p53 gene (Chapter 2.10.2); the primer sequences and annealing temperatures for each reaction are shown in Table 4.1. The PCR products obtained were visualised on polyacrylamide gels (Chapter 2.11) (Figure 4.1), purified and sequencing data obtained (Chapter 2.13). The PCR products of exon 7 were too small for direct sequencing and were therefore cloned into bacterial plasmids prior to sequencing (Chapter 2.12). The sequencing data was compared to the known wild-type sequence of the p53 gene to determine whether mutations were present.

Table 4.1. Oligonucleotide primers used in PCR of clam tumour DNA.

Exon	Annealing temperature	PCR product size /base pairs	Oligonucleotide primers
5	60 °C	259	Forward primer: d(TGCCCTGACTTCAACTCTGTCTCC) Reverse primer: d(CCAGCCCCTGTCTCTCTCCAGCC)
6	65 °C	188	Forward primer: d(GTCCCAGGCCCTCTGATTCCCTC) Reverse primer: d(TAACCCCTCCTCCAGAGACCCAG)
7	60 °C	79	Forward primer: d(ATGTGTAAACAGTTCTTCCATG) Reverse primer: d(CTGACCTGGAGTCTTCCAGTG)
8	60 °C	209	Forward primer: d(ACCTGATTTCCTTACTGCCCTCTTGCTTC) Reverse primer: d(CTTGGTCTCCTCCACCGCTCTTG)

Figure 4.1 Gel electrophoresis demonstrating PCR products for exons 5 (259 base pairs), exon 6 (188 base pairs), exon 7 (79 base pairs) and exon 8 (209 base pairs) of the p53 gene. A 100 base pair molecular marker is shown on the left of the gel.



4.4. Results.

4.4.1. Fluorescence *in-situ* hybridisation of a tumour sample.

Trisomy of chromosomes 8 and 9 and monosomy and trisomy of chromosome 18 were present in the tumour cells (Table 4.2). Only thirty-eight percent of cells were disomic for all three chromosomes studied. Perhaps the most striking feature of the results was the presence of polyploidy in twenty-three percent of the cells (Figure 4.2). Most tumour polyploid cells were either triploid or tetraploid. However, about ten percent of the polyploid cells exhibited gross abnormalities containing up to sixteen times the haploid number of chromosomes (Figure 4.3), representing a severe disturbance of cell function

4.4.2. The p53 gene sequence from tumour DNA.

Sequencing data from exons 5, 7 and 8 were all normal and no mutations were detected. A point mutation was present at the third base of codon 192 in exon 6 in which guanine was substituted by adenine nucleotide (Figure 4.4) indicating that the codon 192 base triplet has been altered from CAG to CAA. However, both of these sequences code for the amino acid glutamine and therefore, this point mutation is silent. No other mutations were found in exon 6.

Table 4.2. Fluorescence *in-situ* hybridisation on a squamous cell carcinoma from a clam ileocystoplasty (abnormal findings highlighted in red).

		Number of cells per 200
Chromosome 8	Monosomy	10
	Diploid cells	128
	Trisomy	13
	Tetrasomy	3
Chromosome 9	Monosomy	6
	Diploid cells	140
	Trisomy	8
	Tetrasomy	0
Chromosome 18	Monosomy	24
	Diploid cells	117
	Trisomy	12
	Tetrasomy	1
Polyploid cells		46

Figure 4.2. Polyploid cells from clam tumour. Chromosomes 8, 9 and 18 are visualised with red, green and aqua probes respectively.

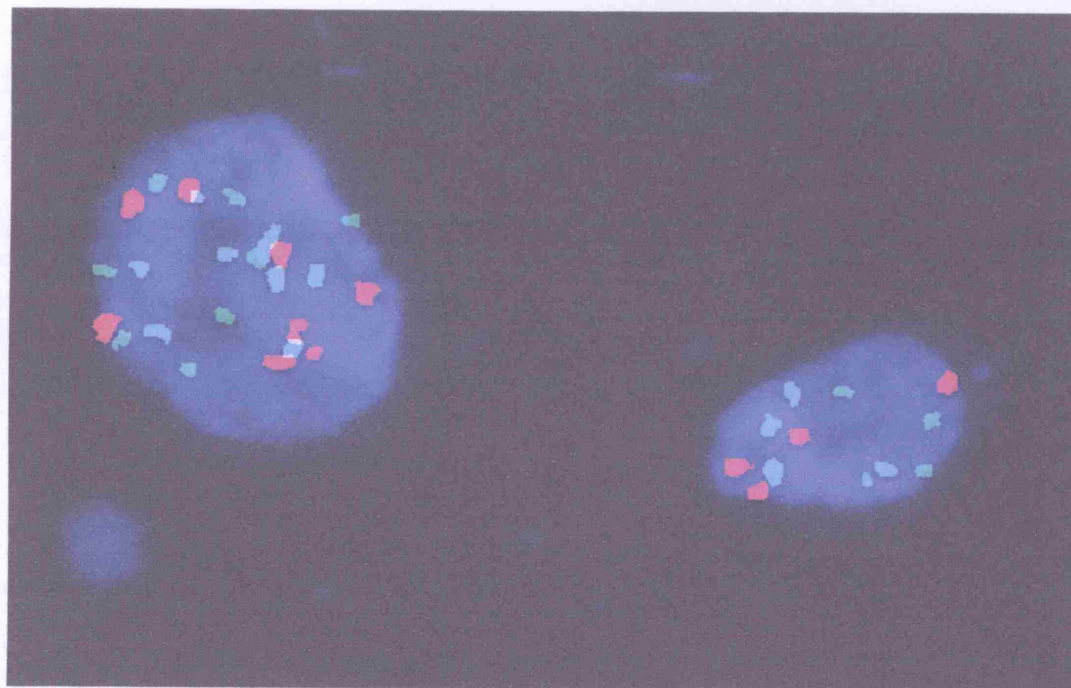


Figure 4.3. Fluorescence *in-situ* hybridisation of tissue from a clam tumour showing a polyploid cell with 16n chromosomes and two normal (diploid) cells. Chromosomes 8, 9 and 18 are visualised with red, green and aqua probes respectively.

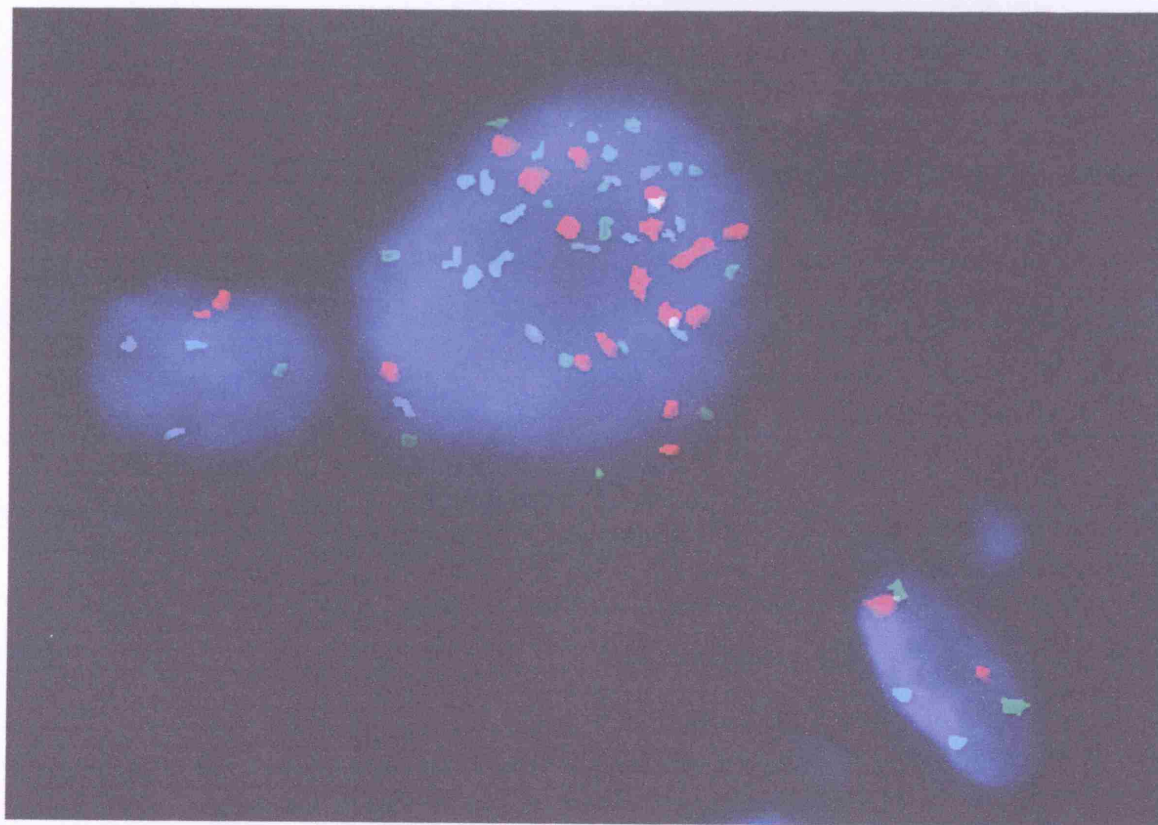
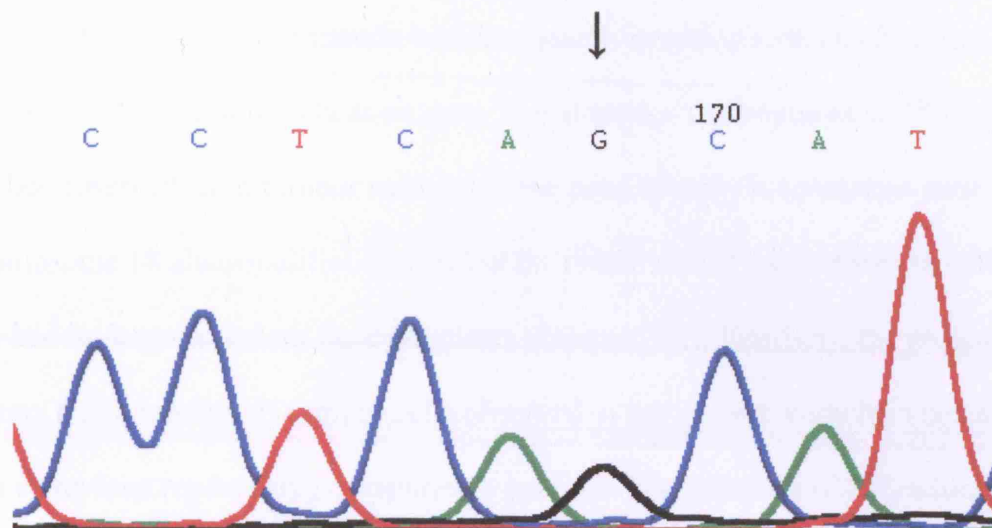
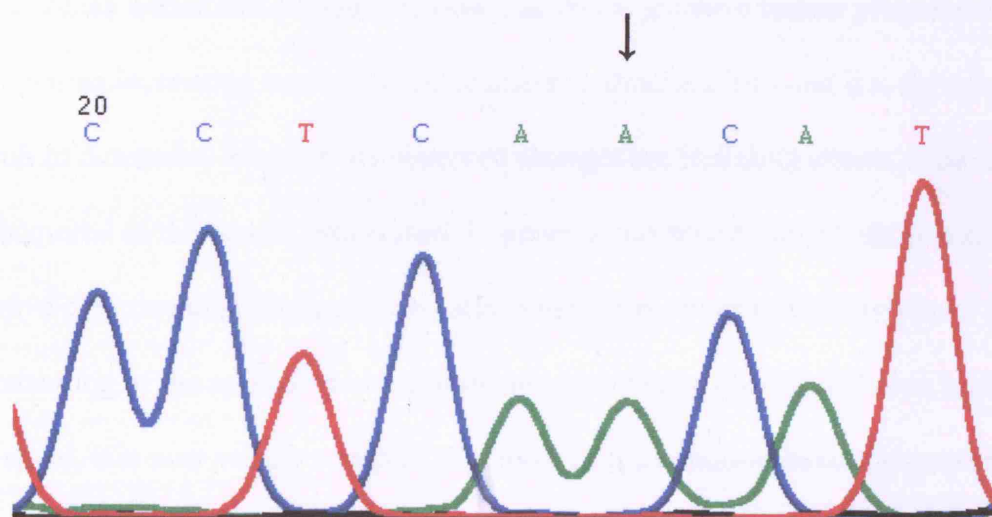


Figure 4.4. Sequencing data demonstrating CAG →CAA mutation at codon 192 of exon 6 in DNA from clam tumour.

Wild-type DNA.



Tumour DNA.



DNA is constructed from a combination of the four nucleotides adenine (A), cytosine (C), guanine (G) and thymine (T). DNA sequencing determines the sequence of nucleotides in a length of DNA. The sequencer detects signals from nucleotides labelled with four different fluorescent dyes. These signals are then plotted on a sequence trace. A DNA trace obtained from the anastomosis of a clam enterocystoplasty is shown. The mutated nucleotide is highlighted with an arrow. A sequence trace from wild-type (normal) DNA is also shown for comparison.

4.5. Discussion.

Tumours that develop within an enterocystoplasty are generally aggressive and have a high mortality (Filmer and Spencer, 1990). In the case described here, the patient presented with haematuria between yearly screening with an advanced tumour and died despite radical surgery. The alteration in chromosome 18 copy number observed from tumour material in the present study is consistent with chromosome 18 abnormalities detected at the enterovesical anastomosis in patients who had undergone a clam ileocystoplasty (Chapter 3.1). Similarly, the presence of trisomy 8 in a number of tumour cells observed in the present study is in agreement with a previous report using comparative genomic hybridisation (CGH) which detected amplification of DNA on the short arm of chromosome 8 in one of two clam tumours (Appanna *et al*, 2000, Appanna, 2004). However, given the extent of the genetic changes detected (thirty-eight percent of the cells were diploid), it seems highly likely that a significant alteration in copy number may be found in other chromosomes within this tumour. Possibly, as this aggressive cancer progressed it developed an increasing number of chromosomal abnormalities and it is therefore difficult to determine whether the observed changes are initiating events or have been acquired as the cancer progressed. In general, advanced cancers show more extensive chromosomal changes than early stages of neoplasia. Although the understanding of the specific molecular events involved in clonal evolution is poorly understood, it is now widely accepted that most of the common human cancers have undergone extensive and variable somatic genetic changes at the time of diagnosis and that this process is a continuing one (Nowell, 1986). Much work has been done with other cancers to characterise tumours by identifying alterations in chromosomal copy number. The ability of cancers to acquire genetic changes has helped to explain the difficulty in finding consistent alterations in common cancers that would permit

the design of specific therapy. Further studies are necessary to determine the initiating events in cancer.

The most striking cytogenetic finding in tissue from the clam tumour in the present study was the large number of polyploid cells which exceeded the prevalence of cells with isolated trisomy 8, trisomy 18 or monosomy 18. Polyploid cells are those cells that contain a number of chromosomes that is an exact multiple of the haploid chromosome number. However, although this definition is adhered to in normal tissue, in the analysis of human cancers polyploidy also includes cells with complex alterations in chromosome number. In the present study only three chromosome pairs were analysed in the clam tumour and if the numbers of all three chromosome pairs were increased in parallel the cell was defined as polyploid. However, because not all chromosomes were visualised using FISH it is possible that some of the cells had complex aneuploid changes rather than being true polyploid cells.

The presence of polyploid cells in cancer tissue probably reflects the accumulation of acquired chromosomal events during the rapid clonal evolution of the tumour. It is also possible that some cells initially became polyploid and then lost chromosomes. The accumulation of alterations in the genetic makeup of tumour cells is thought to lead to aberrant cell cycle control with resulting polyploidy and altered cell behaviour (Holland *et al*, 1998). The nuclei of cells increase in size with increasing polyploidy (Galitski *et al*, 1999) and in the tumour reported here, were much larger than other aneuploid or diploid nuclei. It has been suggested that ploidy dependent repression cyclins acting at the G1 phase of the cell cycle could explain

the larger size of polyploid cells and that polyploid cells have abnormalities in cyclin-mediated cell cycle progression (Galitski *et al*, 1999).

Few papers have been published on the clinical progression of patients with polyploid cancers. Complex genetic abnormalities are known to be associated with a poor prognosis (Parlier *et al*, 1994). Polyploidy has been shown to be an unfavourable prognostic variable in colorectal cancer (Cosimelli *et al*, 1998; Buglioni *et al*, 2001) and patients with polyploid cancers may have an unusually rapid evolution of their disease (Manley *et al*, 1998). Polyploidy has been used as an indicator of prognosis for patients and to identify those who may benefit from more aggressive adjuvant therapy (Cosimelli *et al*, 1998). The presence of polyploidy in the clam cancer in the present study and the aggressive nature of the tumour are consistent with the extremely poor prognosis of polyploid neoplasms. Possibly the presence of polyploidy in tumours arising within an enterocystoplasty may be an indicator of a poor prognosis. Although this approach may be of benefit to some patients, the dismal outcome associated with the aggressiveness of enterocystoplasty tumours (Filmer and Spencer, 1990), suggests that identifying those at risk from an increase rate of tumourigenesis before malignant transformation takes place may be a more successful strategy.

In human cancers where polyploidy is a characteristic finding the cells typically contain three or four times the haploid number of chromosomes (Gunawan *et al*, 1999; Manley *et al*, 1998). There is evidence to suggest that the presence of cells with higher multiples of the haploid chromosome number is associated with a poor prognosis (Borgström *et al*, 1976). In the present study, most tumour polyploid cells were either triploid or tetraploid. However, about ten percent of the polyploid

cells exhibited gross abnormalities containing up to sixteen times the haploid number of chromosomes, representing a severe disturbance of cell function which may be reflected in the clinical outcome. The reason why cancers occurring within an enterocystoplasty are so aggressive is uncertain. It is possible that the apposition of bladder and bowel followed by subsequent cancer initiating events activate a particular gene that is rarely involved with the progression of more common forms of cancer. Another possibility is that factors in the host environment may be potent potentiators of tumourigenesis when cells acquire the neoplastic phenotype. For example, inflammatory mediators present at the enterovesical anastomosis may act as growth factors once a cancer is initiated. Similarly, it is generally accepted that host immune systems have a selective pressure on evolving neoplasms particularly in the early stages of cancer formation and a locally altered immune response at the site of tumour formation may also potentiate tumourigenesis (Klein and Klein, 1985). Many mechanisms have been suggested to be implicated in the initiation, growth and progression of tumours arising within clam ileocystoplasties (Chapter 1.3.3) but as yet there is no conclusive evidence to confirm the role of any mutative tumourigenic factor.

Hitherto, there have not been, as far as we are aware, any reports in the literature of sequencing data from clam tumours. The most commonly described mutations detected in human cancers are those affecting the p53 tumour suppressor gene (Carson and Lois, 1995). Therefore, it appeared logical to investigate the p53 gene in this study. Although, only exons five to eight of the p53 gene were sequenced, eighty-seven percent of all p53 mutations occur in these exons (Greenblatt *et al*, 1994). Furthermore, since thirty percent of the mutations outside exons five to eight are missense, ninety-five percent of all missense mutations occur

in exons five to eight (Greenblatt *et al*, 1994). Perhaps more importantly, only mutations in exons five to eight of the p53 gene result in an increased half-life of the protein product (Kroft and Oyasu, 1994). Most p53 missense mutations are located in the evolutionary highly conserved central DNA binding domain of p53 which is encoded by the four exons studied here (Walker *et al*, 1999).

In the present study, a point mutation of the third base of codon 192 in exon 6 of the p53 gene was detected in the clam tumour indicating that wild-type codon CAG has been mutated to the CAA. Since both CAG and CAA encode for the amino acid glutamine this is a silent mutation. Silent mutations are those in which a nucleotide has been altered to produce a synonymous codon without amino acid change. According to the International Agency for Research on Cancer (IARC) p53 mutation database (www.iarc.fr) which tabulates the mutations in the p53 tumour suppressor gene recorded in peer-reviewed journals (Hainaut *et al*, 1998), 4.9 percent of all p53 mutations are silent mutations (Hernandez-Boussard *et al*, 1999).

It has been suggested that nonsense, missense and even translationally silent mutations can inactivate genes by inducing splicing machinery to skip mutant exons (Cartegni *et al*, 2002). Translationally silent mutations are normally considered to be neutral. Although this assumption may be correct, supporting evidence is required from characterisation of messenger ribonucleic acid (RNA), since mutations that have an effect on splicing modulation are likely to have a marked effect on the translated product. At present most databases contain mutation data that are primarily or exclusively derived from genomic data.

There is increasing evidence that many human diseases harbour exonic mutations that affect splicing of translated nucleotides to produce messenger RNA (Cartegni *et al*, 2002). Inaccurate recognition of exon-intron boundaries or the failure to remove an intron generates aberrant messenger RNA that is either unstable or codes for defective or deleterious proteins. Splicing signals are a frequent target of mutations in genetic disease and cancer and it has been estimated that approximately fifteen percent of point mutations that result in a human genetic disease cause RNA splicing defects (Krawczak *et al*, 1992). Most splicing mutations affect the standard splicing signal and lead to skipping of the neighbouring exon. Occasionally the mutations create an ectopic splice site or activate a cryptic splice site changing the splicing pattern of the mutant transcript. Exon skipping associated with silent mutations has been frequently observed (Cartegni *et al*, 2002). For mutations that are translationally silent to result in exon skipping they must be acting at the RNA level. These mutations probably alter *cis*-elements that are important for correct splicing. *Cis*-elements are combinations of short sequences of nucleotides usually located in the immediate upstream region of the gene and serve to initiate transcription and it is generally accepted that *cis*-acting regulatory sequences control the activity of a gene only when it is part of the same DNA or RNA molecule. Silent mutations are probably underreported because they might be incorrectly assumed to be neutral polymorphisms that do not merit further classification. However, there is increasing evidence that this may not be the case and further work is required to define the structure and function of the messenger RNA product of tumour DNA harbouring silent mutations.

The frequency of silent p53 mutations for each codon has been determined by analysis of the IARC tumour database (Strauss, 2000). Regions of a gene that are

more likely to harbour mutations are considered 'hot spots' (Benzer, 1961).

Approximately one third of p53 silent mutations occur at hot spots the remainder are randomly distributed. The majority of silent mutations in the p53 gene are therefore randomly selected and are neutral but their frequency is an indication of the amount of mutation that occurred during the development of a particular tumour. A 'driver' mutation is required to produce a tumour and this mutation may be in p53 or in some other gene that is part of the p53 cascade. Approximately, two thirds of the silent mutations in the p53 gene are the only mutations in the p53 gene detected in some tumours analysed and are thought to be accompanied by a 'driver' mutation elsewhere in the tumour genome (Strauss, 1998). Different tumour types have been shown to have different frequencies of silent mutations indicating their different developmental history. Tumours of the oesophagus, colon and kidney (Strauss, 2000) exhibit lower percentages and prostate tumours (Chi *et al*, 1994) very high percentages of silent mutations than the overall average for all cancers.

Hot spot mutations in cancers represent protein alterations that provide a selective growth advantage to the cell and are more likely to result in tumourigenesis. The location of hot spot mutations usually denotes a functionally critical amino acid (Walker *et al*, 1999). It is likely that the silent mutations occurring at hot spots confer a growth advantage, either by the previously described mechanisms of altered messenger RNA splicing or by some other further undetected mechanism. The precise role of silent mutations is therefore speculative and studies are required to determine the protein products of mutated tumour genes.

Hot spots for silent mutations in the p53 gene have been described at eight codons: numbers 137; 140; 153; 211; 226; 247; 293; and 299 (Strauss, 2000). In the

present study a mutation at codon 192 was observed in the clam tumour which is not recognised as a hot spot for silent mutations. The precise nature of the significance of this mutation on tumour cell function is uncertain since the messenger RNA protein product has not been characterised. However, it is likely that the mutation at codon 192 is a neutral mutation, which does not play an important role in the development of clam tumours but reflects the increased mutability of the tumour DNA due to a 'driver' mutation elsewhere in the tumour genome.

In the present study, two main genetic abnormalities have been identified, namely polyploidy which may be the underlying genetic abnormality that results in enterocystoplasty tumours following an aggressive clinical course and the presence of a silent p53 mutation which may reflect the increased mutability of the tumour DNA. Further investigation of the genetic abnormalities arising in clam tumours and biopsies in augmentation cystoplasties may suggest further cytogenetic changes or mutations in clam tumours that may help identify those patients with an enterocystoplasty who will go on to develop tumours. As other clam cancers occur, where possible tumour material should be obtained for genetic analysis.

5. Discussion.

The clam enterocystoplasty has revolutionised the lives of those patients with an overactive bladder refractory to non-surgical management. Although the procedure is associated with considerable complications and side effects, the improved quality of life after operation would appear to outweigh the adverse factors (Herschorn and Hewitt, 1998) and the majority of patients are satisfied with their treatment. However, there is a small but real risk of tumour formation in the augmented bladder (Filmer and Spencer, 1990, Ali-El-Dein, 2002).

The incidence of tumours in bladders augmented with bowel is currently unknown. The number of malignancies occurring within an augmentation cystoplasty reported in the literature continues to rise. Sixty cases have been hitherto described (Table 1.1) and we are aware of further cases that have occurred elsewhere. We have so far had six cases in our own unit at the University Hospital of Wales, Cardiff. Four cases have previously been described elsewhere (Barrington *et al*, 1997a) and a further case is described in Chapter 4 (Ivil *et al*, 2006). Sadly, during the time this work was performed, one further patient has died from a disseminated tumour arising within a clam ileocystoplasty. In this case a thirty-eight year old woman with spina bifida presented with diarrhoea, due to an enterovesical fistula seventeen years after undergoing augmentation cystoplasty. An inoperable squamous cell carcinoma involving both the bladder and the bowel at the ileovesical anastomosis was found. These cases highlight the potential for cancer formation within an enterocystoplasty and the need to develop techniques that can identify those patients at risk from life-threatening tumours before they occur.

Fluorescence *in-situ* hybridisation (FISH) was used to study tissue from a squamous cell clam tumour (Chapter 4) and identified an unusually large number of polyploid cells (twenty-three percent). Polyploid cells within tumours are thought to arise from the accumulation of chromosomal events during a rapid clonal evolution of a tumour (Holland *et al*, 1998) and the high number of polyploid cells observed in this study indicates that the tumour was genetically unstable. Polyploidy is known to be an unfavourable prognostic marker (Buglioni *et al*, 2001) and is associated with the rapid progression of a cancer (Manley *et al*, 1998). Therefore, the presence of polyploidy within this fatal squamous cell carcinoma in the present study is consistent with the aggressive nature of this tumour. Cancers forming within enterocystoplasties are extremely aggressive with a high reported mortality (Filmer and Spencer, 1990). Five of the six cases of clam tumour at our own unit have died and this emphasises the need to develop suitable methods for identifying those patients at risk of tumour formation early on in the process of tumourigenesis.

A major aim of cancer genetics is to be able to identify early genetic mutations in premalignant tissue which can act as markers of tumour progression (Cairns and Sidransky, 1999) allowing early identification of individuals who may be at a higher risk of tumourigenesis. Such individuals could be enrolled on surveillance programs or be candidates for surgical intervention. The advantage of studying premalignant tissue is that the prognosis is much better when the disease is diagnosed early. However, since the majority of cells in biopsies of premalignant tissue are normal and the number of genetically altered cells that are thought to initiate tumour formation are small, detection of abnormalities is difficult. Both FISH and the RSM assay are ideally suited for the identification of rare genetic changes. FISH allows

nucleus-by-nucleus analysis of absolute copy number for any given chromosome-specific probe and is therefore a highly sensitive technique for demonstrating chromosomal losses or gains relative to the overall genome of interest. The RSM assay detects rare mutations in the presence of a vast excess of background unmutated wild-type sequences by combining the use of restriction enzymes and the polymerase chain reaction. Therefore, both of these methodologies are suitable for highlighting genetic changes occurring as early events in tumourigenesis.

The purpose of this study was to further investigate mutations in patients who have undergone a clam ileocystoplasty using FISH to confirm instability at the enterovesical anastomosis and to identify more precisely the genetic changes which may predispose to tumour formation in the augmented bladder. Metaphase spreads are not easily obtainable from biopsy material and therefore interphase cytogenetics was performed on touch sample interphase preparations using fluorescent centromeric probes. A potential limitation of FISH is in defining chromosome loss since even when the copy number distribution in a test sample is compared with controls, nuclei with single signals may reflect an impaired hybridisation frequency which is inherent to the test sample. In this study we have attempted to minimise this possibility by using control samples from the same patients.

The results of the present study indicate that chromosomal numerical abnormalities were present at the enterovesical anastomosis following a clam ileocystoplasty in all patients studied. In contrast, no chromosomal numerical abnormalities were observed in the native bladder remnant. In particular, aneuploidy of chromosome 18 was present in thirteen out of fifteen patients and appears to be a good marker for detecting genetic instability at the enterovesical anastomosis.

Previous studies suggest that many mechanisms contribute to an altered homeostasis at the bladder bowel interface which may possibly predispose to chromosomal abnormalities and hence tumour formation (Chapter 1.3.3).

In the present study, subtle chromosomal alterations at the enterovesical anastomosis were observed using FISH. Two patients exhibited a two percent incidence of trisomy 18 which would have certainly not been detected by CGH because the latter technique requires that at least sixty percent of nuclei contain the same genetic alteration for an abnormality to be detected (Kallioniemi *et al*, 1994). Abnormalities of chromosomes 8, 9 and 18 were present in less than sixty percent of the cells in every sample studied. The clonal nature of chromosomal abnormalities suggests that urothelial abnormalities are more prevalent adjacent to the site of the original abnormal cell (Nowell, 1976). Harvesting biopsies when no visual changes are apparent is essentially a random process and it is therefore possible that CGH may detect changes in DNA from biopsies with a higher incidence of chromosomal abnormalities.

Chromosomal abnormalities were present in the biopsies taken from the anastomosis of clam ileocystoplasties even though the urothelium at the anastomosis in these patients was essentially histologically normal. The finding of genetic abnormalities in potentially malignant tissue which is histologically normal is not a new observation and loss of heterozygosity has been described in histologically normal epithelium in oesophageal and gastric biopsies following oesophagogastrectomies for the resection of adenocarcinomas (Dolan *et al*, 2000).

In the present study there was no correlation between the number of aneuploid changes and the time between a patient undergoing operation and subsequent biopsy, possibly because the cohort studied was not large enough and the time between operation and biopsy was not great enough to detect such changes (range 6-18 years). It is also possible that local factors are different for each patient and the process of tumourigenesis occurs at a different rate in individual patients since the latent period between operation and presentation of tumour (2-40 years) is very large. Future studies need to be directed at defining the temporal relations between operation and the development of chromosomal alterations at the enterovesical anastomosis in patients who have undergone enterocystoplasty to determine whether the genetic changes increase with time.

Any one or combination of the mechanisms described in Chapter 1.3.3 may be involved in producing aneuploidy in the clam ileocystoplasty. If the pathological processes that result in aneuploidy could be determined then this may allow therapies to be tailored for the treatment of individual cancers. Irrespective of the mechanism responsible for the changes observed in this study, aneuploidy indicates that chromosomal instability is occurring at the anastomosis. Further studies are required to determine the heterogeneity of the chromosomal abnormalities occurring at the enterovesical anastomosis in individual patients using multiple and sequential biopsies to establish which changes are important in the development and progression of tumours.

In the present study aneuploidy was observed at the enterovesical anastomosis but not elsewhere in the native bladder. The results of the present study using FISH are in accord with previous findings using CGH which demonstrated

instability of chromosomes 8, 9 and 18 at the enterovesical anastomosis of patients who had undergone a clam ileocystoplasty (Appanna *et al*, 2000, Appanna, 2004). These observations suggest that the enterovesical anastomosis is inherently genetically unstable resulting in an increased rate of mutagenesis of the epithelium. However, the chromosomal abnormalities identified in the present study do not in themselves identify those patients who have undergone an enterocystoplasty and who are at an immediate risk of developing life-threatening tumours. Nevertheless, the high frequency of aneuploidy at the anastomosis suggests that these chromosomal changes may precede the formation of invasive cancer. Since cancers occurring within clam ileocystoplasties are highly aggressive and have developed rapidly by the time they are diagnosed, a method that predicts tumour formation would be beneficial in the management of these patients. The results of this study suggest detection of chromosomal losses and gains may be a useful marker for tumour formation but this suggestion requires further investigation. Several chromosomal changes have been highlighted in this study. Thus, chromosome 18 aneuploidy is particularly prevalent and is a useful marker of instability. Follow-up of patients is required to confirm whether a particular type of abnormality is associated with tumour formation.

Aneuploidy of chromosome 18 was the most common chromosomal alteration observed in this study suggesting that it may be useful as a marker of genetic instability. Gains and losses of chromosome 18 were observed in a similar number of patients suggesting that non-disjunction is the possible mechanism responsible for the aneuploidy observed in these patients. In two patients both monosomy and trisomy were present whereas in the remainder of the patients one type of cell predominated. It is likely that in patients displaying isolated monosomy

or trisomy, the growth of one population of cells was favoured, either by the influence of the local environment at the anastomosis or by cellular genetic changes occurring at the anastomosis.

Previous work using comparative genomic hybridisation (CGH) identified amplifications on the short-arm of chromosome 18 (18p) at the ileovesical anastomosis of patients who had undergone a clam ileocystoplasty (Appanna *et al*, 2000, Appanna, 2004). It is uncertain whether instability of chromosome 18 observed in the present study using FISH reflects changes on 18p since the two studies used separate patient groups. Abnormalities of 18p have only very rarely been reported to be associated with cancer development. However, loss of heterozygosity of the long-arm of chromosome 18 (18q) has been described in a number of human malignancies and several candidate genes identified, particularly in colon adenocarcinoma (Vogelstein *et al*, 1988) but also in transitional carcinoma (Reznikoff *et al*, 1996).

Sixty to eighty percent of colorectal carcinomas display loss of 18q (Vogelstein *et al*, 1988). Loss of 18q appears to occur late in the adenoma-carcinoma sequence and has been reported to be associated with a poor prognosis (Kern *et al*, 1989, Jen *et al*, 1994). In colorectal tumours the majority of deletions of 18q have been localised to 18q21 which corresponds to the locus of the deleted in colon cancer (DCC) tumour suppressor gene (Fearon *et al*, 1990). Loss of the DCC gene has been shown to be an independent prognostic variable in patients with a colorectal cancer and is associated with a poor outcome (Shibata *et al*, 1996). However, recent evidence has supported the presence of other possible tumour-suppressor genes in the 18q21 chromosomal region which may influence the growth

and progression of colorectal cancer. Two of the genes suggested as being important in the pathogenesis of colorectal cancer are MADR2 and DPC4 which are located within the transforming growth factor β (TGF β) signalling pathway (Gryte, 1997). The TGF β family of proteins has a wide variety of biological functions including growth control, cellular differentiation, embryonal morphology and immunity.

Loss of heterozygosity of chromosome 18q has been found in approximately twelve percent of transitional carcinomas (Reznikoff *et al*, 1996). Allelic loss of one or more 18q loci is associated with muscle-invasive transitional cell carcinoma of the bladder and has been attributed in some cases to deletion of the DCC gene (Brewster *et al*, 1994). However, further work is required to define more precisely the role of aberrations of chromosome 18 in the development and progression of bladder cancer (Knowles, 1999a).

It is not possible to determine whether instability of chromosomes detected in the present study was related to changes in the copy numbers of the long or short arms of chromosome 18. Interphase cytogenetics using centromeric probes, by definition identifies gains and losses of centromeres. In order to ascertain whether 18p or 18q copy number or a complete loss or gain of chromosome 18 was present at the anastomosis of patients who have undergone enterocystoplasty, centromeric probes could be combined with telomeric probes (Fomina *et al*, 2001). Nevertheless, irrespective of whether or not alterations occurred in 18p or 18q, aneuploidy of chromosome 18 determined by FISH appears to be a good marker of instability at the enterovesical anastomosis in patients who have undergone a clam enterocystoplasty. Further prospective, longitudinal studies are required to demonstrate if aneuploidy of

chromosome 18 is a useful prognostic marker of increased risk of tumour formation in augmented bladders.

CGH has been used to identify chromosomal instability on the short-arm of chromosome 8 (8p) in DNA from a clam tumour (Appanna *et al.* 2000, Appanna, 2004). Importantly, in the present study, FISH detected instability of chromosome 8 at the enterovesical anastomosis of two patients with a clam ileocystoplasty. It is of interest that in each case, instability of chromosome 18 was also present. Therefore, aneuploidy of chromosome 8 may be associated with a greater magnitude of genomic instability than aneuploidy of chromosome 18 and it is possible that patients with chromosome 8 instability are at an increased risk of malignant transformation. Furthermore, deletions of 8p have been reported to be present in over fifty percent of muscle-invasive transitional carcinomas. Importantly, only alterations in retinoblastoma and p53 gene function are found with a greater incidence than deletions of 8p in advanced bladder cancer (Knowles, 1999a). Two deleted regions have been identified on 8p in transitional cell carcinoma (Takle and Knowles, 1996) which coincide with deleted regions reported in colorectal cancer (Fujiwara *et al.*, 1993). However, no active tumour suppressor gene has yet been identified on chromosome 8p (Knowles, 1999b). Possible target genes for carcinogenesis located on 8p are POLB, PPP2CB, NAT1 and NAT2, but these have been excluded in transitional cell carcinoma (Knowles, 1999a). Deletion of 8p is present in up to ninety percent of colorectal cancers (van der Bosch *et al.*, 1992) and is indicative of both recurrence (Zhou *et al.*, 2002) and invasiveness (Keleman *et al.*, 1994). However, the significance of these findings with respect to tumour growth and progression is difficult to assess. Since deletion of 8p is associated with a poor outcome in both urothelial (Knowles, 1999a) and colorectal carcinomas (van der Bosch *et al.*, 1992), it

is possible that changes in this chromosome rather than abnormalities of chromosome 18 may be useful as a genetic marker that will predict which patients will develop an invasive carcinoma. In this study chromosome 18 has been shown to be a good marker for genetic instability. It is of interest that the two patients who demonstrated both loss and gain of chromosome 18 also displayed chromosome 8 instability, one patient showing a loss and the other a gain of chromosome 8 copy number (Table 3.5). These two patients in particular should be followed up closely to determine whether tumour formation occurs more rapidly than in the other patients studied.

Amplification of the long-arm of chromosome 8 (8q) occurs in about forty-five percent of transitional cell carcinomas (Knowles, 1999a). The *c-myc* oncogene is located on chromosome 8q and is overexpressed in some high grade transitional carcinomas (Kotake *et al*, 1990) but does not correlate with patient survival or tumour progression (Lipponen, 1995). Furthermore, the expression of *c-myc* does not correlate with amplifications observed on chromosome 8q in transitional carcinoma and therefore it is uncertain whether *c-myc* or another gene is implicated in tumourigenesis (Sauter *et al*, 1995). However, in colorectal cancer *c-myc* gene amplification has been shown to be associated with tumour aggressiveness (Obara *et al*, 2001).

In the present study, FISH identified a loss of chromosome 9 copy number at the enterovesical anastomosis in nine out of fifteen patients. Possibly, loss of chromosome 9 at the anastomosis of patients who have undergone an enterocystoplasty is a non-specific event, then this chromosomal abnormality may not be useful in predicting which patients will go on to develop tumours. Loss of

heterozygosity of the long and short arms of chromosome 9 are the most commonly reported deletions in bladder carcinoma and are often the only genetic aberrations in superficial transitional carcinomas (Brandau and Böhle, 2001). Moreover, in most cases the entire chromosome (both arms) is missing. These observations suggest that the inactivation of a gene or genes on chromosome 9 may be an important step in the initiation of tumourigenesis in superficial bladder tumours (Brandau and Böhle, 2001). In the present study, FISH identified early losses of chromosome 9 in patients who had undergone a clam ileocystoplasty. However, alterations in chromosome 9 copy number are a late event in the natural history of carcinoma *in-situ* of the bladder and in this situation are thought to contribute to invasion (Spruck *et al*, 1994). Five areas have been identified on the long-arm of chromosome 9 (9q) which may contain candidate genes for carcinoma formation but no specific gene has yet been identified (Knowles, 1999a). CDKN2 is located at the 9p21 region on the short-arm of chromosome 9 (9p) and has been identified as a possible candidate gene for tumourigenesis. Loss of heterozygosity of 9p21 occurs in all bladder cancers independent of stage and grade and therefore this genetic anomaly is considered an important step in the development of these tumours (Brandau and Böhle, 2001). CDKN2 encodes for the protein p16 which is an inhibitor of cyclin-dependent kinase 4 which phosphorylates the retinoblastoma gene product. Loss of p16 results in uncontrolled phosphorylation of the retinoblastoma protein by complexes of cyclin and cyclin-dependent kinase prolonging the cell cycle's transition into S phase. In contrast to bladder cancer, there is less convincing evidence to implicate chromosome 9 in the growth and progression of colorectal carcinoma. A loss of 9p has been associated with an early age of onset of colorectal carcinoma (Weber *et al*, 1999) and there is one report of a high incidence of 9q rearrangements, particularly breakpoints at 9q22, in colorectal carcinoma (Konstantinova *et al*, 1991).

It is unlikely that every patient with an enterocystoplasty will form a life-threatening tumour since the latent period between operation and the appearance of a clam tumour varies between two (Lane and Shah, 2000) and forty years (Sato *et al*, 2000, Ulmer *et al*, 2000). Therefore, the finding of generalised chromosomal changes in the present study appears to be a non-specific marker of genetic instability and may not be helpful in identifying those patients at an immediate risk of developing a life-threatening tumour. It is uncertain whether cancers occurring within an enterocystoplasty are of bladder or bowel origin (Murray *et al*, 1995, Barrington *et al*, 1997a). The chromosomal changes identified in this study do not indicate whether tumours occurring within an augmentation cystoplasty originate in bladder or bowel, or are a separate entity. However, the results of this study suggest that aneuploidy of chromosome 18 may be a good marker for genomic instability at the enterovesical anastomosis and in addition, suggest that aneuploidy of chromosome 8 or chromosome 9 may be a more discerning marker of those patients who will develop a life-threatening cancer. In particular, aneuploidy of chromosome 8 appears to be associated with more severe genomic instability at the bladder-bowel interface. Polyploidy was not identified in any of the surveillance biopsies taken from the enterovesical anastomosis. However, polyploidy was the salient finding in the tumour material analysed using FISH (Chapter 4). These observations suggest that if polyploidy is demonstrated in biopsies obtained from patients with a clam enterocystoplasty, these patients should be carefully followed up. Longitudinal studies are required to determine whether any particular chromosomal change is associated with cancer formation at the enterovesical anastomosis in patients following an augmentation cystoplasty.

The RSM assay is a relatively new technology, first described in 1990 (Parry *et al.*, 1990) and has recently been used to detect early p53 mutations in several premalignant conditions including inflammatory colon tissue (Ambs *et al.*, 1999, Perwez Hussain *et al.*, 2000), Barrett's oesophagus (Jenkins *et al.*, 2003) and gastritis (Morgan *et al.*, 2003). Further studies and long-term follow up are required in order to confirm if the described changes are associated with subsequent cancer formation. Due to the pivotal role of the p53 gene in tumour suppression, loss of p53 function may well be indicative of tumourigenesis. Recently, a case has been described of a patient with low-grade dysplasia of the oesophagus containing a p53 mutation identified by RSM analysis, which progressed to adenocarcinoma within thirteen months of follow-up (Jenkins *et al.*, 2003). However, at present it is not clear whether patients with p53 changes as early mutational events progress to cancer more rapidly than patients without p53 mutations. Nevertheless, the RSM assay may prove useful in identifying patients who are at an increased risk from invasive tumours, but further studies are required to substantiate these suggestions. In particular, the latent period between the detection of p53 changes and subsequent cancer formation requires further investigation.

The results of this study indicate that the RSM assay can detect rare p53 mutations at the ileovesical anastomosis in non-cancer patients who have undergone a clam ileocystoplasty confirming that this anastomosis is genetically unstable. The RSM method appears well suited for scanning hotspot codons of p53, which would be undetectable by less sensitive contemporary techniques. Furthermore, the results indicate that clonal expansion of p53 mutated cells is occurring in patients with an ileocystoplasty whose bladders are normal when visualised with endoscopy. If early mutations of the p53 tumour suppressor gene are validated as a clinically useful

biomarker for cancer progression this may allow the frequency of tumour surveillance within a screening program to be optimised for the early detection of patients at risk of cancer formation within ileocystoplasties.

In the present study, the RSM assay identified mutations of the p53 gene at the anastomosis in seven out of thirty-eight patients who had undergone an enterocystoplasty (Chapter 3.2). However, these changes are unlikely to be the first mutations to occur in these patients, but rather reflect the general genetic instability of the enterovesical anastomosis since the DNA encoding the p53 gene forms only a tiny part of the human genome. Furthermore, in the present study only five restriction sites were investigated by RSM and therefore it is possible that many other mutations were also present in the patients studied because of the genetic instability of the enterovesical anastomosis. Moreover, at least ten identical copies of a mutation resistant to enzyme digestion are required to produce a visible band on a gel after RSM analysis (Steingrimsdottir *et al*, 1996, Jenkins *et al*, 2001). FISH has demonstrated that many of the patients who have undergone an enterocystoplasty exhibit genetic instability at the bladder-bowel interface (Chapter 3.1). The RSM assay is more selective than FISH and may identify those patients with a greater number of mutational events. Therefore, it is possible that the RSM assay may be more useful than FISH in determining those patients who are at a higher risk of tumour formation but this suggestion needs to be substantiated by longitudinal studies of patients following an augmentation cystoplasty.

Seven patients were identified with p53 mutations at the ileovesical anastomosis. In two patients, a silent mutation was detected. These patients are of

interest as silent mutations do not alter the amino-acid sequence of p53 and therefore do not undergo clonal expansion. Silent mutations accumulate with time due to increased mutagen exposure (Strauss, 2000). At least ten identical mutations are required to obtain an identifiable PCR product with the RSM assay (Steingrimsdottir *et al.*, 1996; Jenkins *et al.*, 2001). Therefore, at least ten separate mutational events must have taken place for silent mutations to be identified in the present study. The majority of silent mutations in the p53 gene are randomly selected and are neutral (Strauss, 1998). Silent mutations usually occur in the presence of a 'driver' mutation which may be in p53 or in another gene that is part of the p53 cascade. It is unlikely that the silent mutations highlighted by this study will initiate cancer. However, these mutations are indicative of genomic instability at the enterovesical anastomosis and therefore indicate patients at an increased risk of developing a life-threatening tumour.

Genetic instability was confirmed at the ileovesical anastomosis of seven of the patients who had undergone a clam ileocystoplasty. However, this study cannot confirm that the other patients do not have any p53 mutations present. By its very nature, RSM analysis can only detect mutations at targeted hotspots. By increasing the number of codons targeted, the sensitivity of the RSM assay can be increased but mutations outside these areas will inevitably be missed. It is outside the scope of this study to provide analysis of every codon of the p53 gene. We have targeted hotspots in the p53 gene frequently implicated in tumour formation and have shown that RSM methodology can provide a rapid method for screening patients for rare p53 mutations which may be indicative of future tumour formation.

Six of the seven mutations detected in this study were mutations of exon 7.

RSM studies have been carried out to determine the effects of reactive oxygen species on the p53 gene in human cells and also to detect rare p53 mutations in premalignant tissues where inflammation is thought to contribute to cancer formation. The majority of these rare p53 mutations were also found in exon 7 (Jenkins *et al*, 2001; Morgan *et al*, 2003; Jenkins *et al*, 2003). Therefore, mutations of exon 7 may prove particularly useful as indicators of instability at the enterovesical anastomosis and highlight those patients at an increased risk of carcinoma formation.

The present study has characterised the early p53 mutations identified at the ileovesical anastomosis of patients who have undergone a clam ileocystoplasty. Although it is not possible to determine precisely the mechanisms responsible for these changes, the type of p53 mutation found may implicate certain carcinogenic agents. Four of the mutations identified by this study were GC → AT transitions, three of them occurring at CpG sites. GC → AT transitions at CpG sites are typical findings after either N-nitrosamine exposure (Saffhill *et al*, 1985) or after oxidative damage to DNA (Feig *et al*, 1994). CpG sites are those where a cytosine nucleotide lies adjacent to a guanine nucleotide joined by the sugar-phosphate backbone in the DNA helix and in this context the carbon atom is often methylated at the 5-carbon position. CpG sites are known to be susceptible to spontaneous deamination of methylated cytosine leading to the conversion of methyl-cytosine to thymine (Pfeiffer, 2000) a process thought to be enhanced by exposure to mutagens or inflammation (Ambs *et al*, 1999). In this study, one patient with a complex mutation at codon 248 was identified. Multiple mutations of the p53 gene is characteristic of N-nitrosamine induced carcinogenesis (Ohgaki *et al*, 1992). N-nitrosamines are implicated in tumourigenesis of schistosomiasis induced squamous cell carcinoma of

the bladder and multiple mutations of the p53 gene have been observed in thirty percent of these cancers (Badawi, 1996).

The results of this study confirm previous reports (Appanna *et al*, 2000, Appanna, 2004) suggesting that the ileovesical anastomosis is genetically unstable in some patients who have undergone an augmentation ileocystoplasty. Furthermore, in this study a subset of patients who had undergone a clam ileocystoplasty, p53 mutations were identified in a small number of cells. The detected mutations, GC → AT transitions, resemble those induced by reactive oxygen species and N-nitrosamines, suggesting a possible role of these agents in the aetiology of carcinoma formation in clam ileocystoplasty. It is not clear whether patients displaying p53 changes as early mutational events progress to cancer more rapidly than patients without p53 mutations. However, p53 mutations have been shown to be markers of anastomotic instability in clam ileocystoplasties of some patients, irrespective of whether the specific changes identified lead to a malignant tumour. Therefore, p53 mutations identified by the RSM assay at the enterovesical anastomosis of patients who have undergone a clam ileocystoplasty may prove useful as biomarkers for instability and cancer risk. Further studies are required to determine the possible temporal relationships between the detection of early p53 changes and tumorigenesis.

In the present study, FISH and RSM were only performed on cells or DNA obtained from one bladder biopsy taken from each patient. Therefore, it is uncertain whether the observed changes are representative of the entire enterovesical anastomosis in the patients investigated in this study. It is possible that mutational events are randomly distributed along the length of the anastomosis and that there

may be a variety of mutations present throughout its length. Further work is required to determine whether the changes observed in this study are consistently found when multiple biopsies of the enterovesical anastomosis are analysed from the same patient. Often there is a need for multiple biopsies in cancer management. Thus, it is recognised that multiple biopsies are useful in staging transitional cell carcinoma of the bladder (Maruniak *et al.*, 2002) and in identifying adenocarcinoma of the oesophagus within areas of dysplastic epithelium (DeMeester and DeMeester, 1999). If the genetic changes at the anastomosis are sporadic, it will be important to determine the optimal number of biopsies that should be taken from the anastomosis for the technique to be suitable for tumour surveillance in patients who have undergone an enterocystoplasty.

The genetic changes highlighted by FISH and RSM may be dependent on each other. P53 loss is thought to be very important in cancer development because of the pivotal role in maintaining genomic instability. It is well known that p53 loss leads to increased spontaneous mutation rates (Havre *et al.*, 1995), disordered replication, chromosome instability (Bouffler *et al.*, 1995) and aneuploidy (Fukasawa *et al.*, 1996). Furthermore, p53 mutations precede aneuploidy in the formation of certain cancers (Neshat *et al.*, 1994, Prevo *et al.*, 1999). In this study, separate biopsies were used for touch preparations for FISH experiments (Chapter 3.1) and obtaining DNA for RSM analysis (Chapter 3.2). It is not known whether genetic abnormalities in a given biopsy are found elsewhere along the anastomosis of a clam ileocystoplasty and therefore in this study it is uncertain whether the findings in FISH and RSM assay experiments are associated. Future studies using matched biopsy samples are required to determine the relationship between aneuploidy and

p53 mutations at the enterovesical anastomosis of patients who have undergone an enterocystoplasty.

In the present study, genetic changes were identified at the enterovesical anastomosis of patients who had undergone a clam ileocystoplasty. However, it is not possible from these observations to determine whether the changes observed with either FISH or the RSM assay are essential to the development of a tumour. Nevertheless, the detection of these genetic changes confirms previous observations suggesting that the enterovesical anastomosis is genetically unstable. Genetic instability has been shown to lead to cancer formation in a variety of tumour types (Marx, 2002). Therefore, by demonstrating genetic changes at the enterovesical anastomosis, the results of this study provides further evidence that patients with an enterocystoplasty are susceptible to the development of tumours.

Controversy exists as to whether carcinomas in enterocystoplasties arise from bowel epithelium (Murray *et al*, 1995) or urothelium (Barrington *et al*, 1997a). Since bladder and bowel epithelium contribute to the anastomosis, it is possible that either tissue could become genetically unstable and give rise to tumour formation. Clam tumours may occur due to abnormalities of a gene or genes unique to this unusual cancer. Alternatively as has been discussed previously, genetic alterations previously identified in bladder or bowel cancer may be helpful in identifying candidate genes in clam tumours. Clam cancers can be a variety of histological types and adenocarcinomas, squamous cell tumours, transitional cell carcinomas (Filmer and Spencer, 1990), two small cell carcinomas (Golomb *et al*, 1989, Bruyneel *et al*, 2004) and a leiomyosarcoma (Egbert *et al*, 1980) have been described. The genetic origin of these tumours is unknown and the varying pathology possibly results from

different separate genetic mutations, different tissue of origin or a combination of these effects.

Recently, attention has focussed on genomic instability and its role in the development of tumours (Kiberstis and Marx, 2002). Cancer is a disease of impaired genomic stability. The molecular forces that maintain genome integrity and sense altered chromosome structure are invariably subverted in cancer cells (Maser and DePinho, 2002). Both FISH and the RSM assay have identified genomic instability at the ileovesical anastomosis of patients who have undergone a clam ileocystoplasty and may prove useful techniques in identifying those patients at risk from the development of life-threatening tumours.

Cancers are initiated by the accumulation of genetic abnormalities. Genomic instability is thought to be essential in this process and makes the cancer cell genome more susceptible than that of normal cells to develop the various cellular abnormalities that characterise the tumour phenotype (Marx, 2002). Most cancer cells exhibit signs of genome instability ranging from elevated mutation rates to gross chromosomal rearrangements and alterations in chromosomal number. Furthermore, the increased incidence of neoplasia in patients with genetic disorders and genomic instability provides further evidence of the importance of genetic abnormalities in cancer formation (Hoeijmakers, 2001).

Controversy exists as to what type of instability results in the changes involved in tumourigenesis and whether genomic instability causes malignancy or results from malignant transformation (Kiberstis and Marx, 2002). It has been proposed that cancer cells have a 'mutator phenotype' that makes them more prone

to acquiring mutations involving short segments of DNA (Loeb and Loeb, 2000). However, it has been suggested that much bigger genetic changes that involve losses or gains of whole chromosomes or shuffling of large segments either within or between chromosomes are required for tumour formation (Rasnick and Duesberg, 1999). Alternatively, cancer cells may have the same tendency for genomic instability as normal cells but because they divide more often, they accumulate more mutations (Marx, 2002). Whatever the mechanism of genomic instability in clam cancers, the results of the present study using FISH and the RSM assay clearly demonstrated genetic changes at the anastomosis of patients who had undergone a clam ileocystoplasty. Possibly therefore, the genetic changes observed at the enterovesical anastomosis in the present study may in time promote tumourigenesis but this hypothesis requires confirmation.

Inflammation has been implicated as an early event in the development of several cancers (Shacter and Weitzman, 2002) and is thought to participate in tumourigenesis at the anastomosis between urothelium and bowel (Filmer and Spencer, 1990). Inflammatory mediators, including prostaglandins, growth factors and free radicals, are produced by inflammatory leukocytes, including neutrophils, monocytes, macrophages and eosinophils. Chronic exposure to these inflammatory mediators results in increased cell proliferation, mutagenesis, oncogene activation and angiogenesis and ultimately results in the proliferation of cells that have lost normal growth control. In general, the longer the inflammation persists, the higher the risk of cancer (Shacter and Weitzman, 2002). The incorporated bowel segment of a clam cystoplasty is frequently observed to be inflamed at endoscopy (Nurse and Mundy, 1989b) and may render the enterocystoplasty susceptible to malignant

change. Furthermore, cystoplasties which are both infected and inflamed have elevated urinary nitrosamine levels (Creagh *et al*, 1997).

Non-steroidal anti-inflammatory drugs (NSAIDs) such as aspirin inhibit cyclo-oxygenase (COX), the rate-limiting enzyme in the conversion of arachidonic acid to prostaglandins. Prostaglandins are present in most human tissues and have been implicated in the formation of tumours (Taketo, 1998). Prostaglandin synthesis, which can be stimulated by free radicals, can further catalyse the formation of free radicals as part of the body's defence mechanisms against foreign bacteria (Shacter and Weitzman, 2002). Therefore, the formation of prostaglandins and free radicals is interdependent. Two COX genes have been cloned which have similar enzyme activities. COX-1 is considered to be a house keeping gene and prostaglandins synthesised via the COX-1 pathway are thought to be responsible for the cytoprotection of the gastrointestinal tract. In contrast COX-2 is involved in the production of inflammatory mediators and has been linked to carcinogenesis. At least five mechanisms by which COX-2 contributes to tumourigenesis and the malignant phenotype of tumour cells have been identified including inhibition of apoptosis, increased angiogenesis, increased invasiveness, modulation of inflammation and conversion of procarcinogens to carcinogens (Dempke *et al*, 2001). NSAIDs inhibit the actions of both COX-1 and COX-2. Epidemiological studies have documented a forty to fifty percent reduction in incidence of colorectal cancer in individuals taking NSAIDs (Smalley and DuBois, 1997). Furthermore, in patients with familial adenomatous polyposis, NSAIDs elicit regression of colonic adenomas (Giardiello *et al*, 1996).

Selective inhibitors of COX-2 have been developed that initially appeared to have fewer side effects than traditional NSAIDs when used as anti-inflammatory or analgesic agents (Dannenberg and Zakim, 1999, Fosslien, 2000). Several studies have provided evidence that inhibition of COX-2 may be an effective strategy for preventing colorectal cancer (Dempke *et al*, 2001). In addition, COX-2 inhibitors have been demonstrated to inhibit tumourigenesis in a wide range of experimental animal models (Dempke *et al*, 2001). Consequently, it has been suggested that the conditions provided by a chronic inflammatory environment are so essential to the progression of the neoplastic process that therapeutic intervention aimed at inhibiting inflammation may have a major role in reducing the incidence of many cancers (Byrne and Dalglish, 2001). Since inflammation, particularly in the presence of bacteriuria has been suggested to play a role in tumourigenesis in enterocystoplasties (Filmer and Spencer, 1990), COX-2 inhibitors may be useful in preventing cancer formation in patients who have undergone a bladder augmentation and this suggestion needs to be substantiated in further studies. Recently, there has been concern regarding the safety of COX-2 inhibitors. In September 2004, Merck, Sharp and Dohme voluntarily withdrew rofecoxib from the market due to an increased risk of cardiovascular death in patients taking the drug long-term (Davies and Jamali, 2004). Similarly, there are concerns regarding the safety of long-term use of other COX-2 inhibitors. However, the cardiovascular effects of COX-2 inhibitors is not uniform (Kimmel *et al*, 2005) and further studies are required to determine whether all COX-2 inhibitors are associated with an increased cardiovascular risk or whether the risk only occurs with particular drugs.

A number of carcinogens have been implicated in tumourigenesis within enterocystoplasties but their precise role has not been established (Chapter 1.3.3).

Oxidative DNA damage plays a major role in tumourigenesis and is thought to be implicated in the development of up to fifty percent of human cancers (Beckman and Ames, 1997). Many types of oxygen radical are produced by inflammation, including the superoxide radical and hydrogen peroxide, both of which lead to the formation of hydroxyl radicals which are thought to initiate most of the observed DNA changes observed when oxygen radicals are formed (Henle and Linn, 1997). Reactive oxygen species generate up to twenty different types of DNA adducts (Jaruga and Dizdaroglu, 1996). One of the most important of these adducts in the induction of mutations is 5-hydroxy-2'-deoxycytidine (Feig *et al*, 1994) which is responsible for reactive oxygen species-induced GC → AT transitions (Jaruga and Dizdaroglu, 1996). Another adduct, 8-hydroxy-deoxyguanosine represents approximately ten percent of oxidative DNA adducts (Jaruga and Dizdaroglu, 1996) and induces GC → TA transversions by misreplication (Cheng *et al*, 1992).

The enterovesical anastomosis is constantly bathed in urine and urinary nitrosamines have been suggested as possible carcinogens (Nurse and Mundy, 1989b). Nitrates are normal constituents of human urine and certain bacteria can metabolise nitrate to nitrosamines which are potent carcinogens (Magee and Barnes, 1956, Calmels *et al*, 1985). Elevated nitrosamine levels have been reported in the urine of patients who have undergone an enterocystoplasty (Nurse and Mundy, 1989b). In particular, urinary nitrosamine levels are increased when bacteriuria exists along with chronic inflammatory changes in the cystoplasty (Creagh *et al*, 1997). These observations suggest that patients with an inflamed cystoplasty and an accompanying infection are most at risk from malignant change.

The alkylating agents that arise from N-nitroso compounds can form a wide range of adducts. However, alkylation at the O⁶ position of guanine is probably the most significant with respect to tumourigenesis since it has been associated with the cytotoxic, mutagenic, carcinogenic and other biological effects of alkylating agents (Saffhill *et al.*, 1985). DNA adducts commonly occurring after alkylation at the O⁶ position of guanine include O⁶-alkylguanine and O⁶-methylguanine. Once present in DNA, O⁶-adducts cause an alteration of its hydrogen binding properties. Subsequent cycles of DNA synthesis allow a misincorporation of thymine and then fix the lesion as a GC → AT transition mutation ultimately leading to an amino-acid exchange (Badawi *et al.*, 1995). This molecular event is one of many that are known to occur during tumourigenesis (Badawi, 1996). A predominance of GC → AT transitions in the p53 gene is a characteristic of the molecular events arising from N-nitrosamine exposure and a high proportion of tumours resulting from N-nitrosamine exposure carry multiple p53 mutations (Ohgaki *et al.*, 1992).

The early p53 mutations identified by the RSM assay have been characterised by sequencing the polymerase chain reaction (PCR) products obtained. The possible mechanisms of DNA damage can be hypothesised by comparing the types of mutation identified with the genetic changes known to occur after exposure to particular carcinogens. Both nitrosamines and free oxygen radicals are implicated in the pathogenesis of cancers occurring within augmentation cystoplasties (Filmer and Spencer, 1990). In the present study, four (sixty-seven percent) of the six mutations characterised by sequencing were GC → AT transitions, three of them at CpG sites. GC → AT transitions are a feature of both nitrosamine and oxygen radical damage and therefore these mutations could have resulted from exposure to either N-nitrosamines or inflammatory mediators. A CGG → CGC transversion that was

identified at codon 248 in the present study was part of a complex mutation (Figure 3.14) and such events are commonly described after nitrosamine exposure (Ohgaki *et al.* 1992). Hence, it is possible that reactive oxygen species and N-nitrosamines may have been responsible for the changes found at the ileovesical anastomosis in the patients investigated in this study and further studies are required to substantiate this suggestion.

The identification of patients with a genetically unstable anastomosis presents a dilemma as to how these patients should be managed. Routine tumour surveillance (screening) of patients who have undergone an enterocystoplasty is practised by many clinicians (Shaw and Lewis, 1999). However, none of the tumours described in the literature have been detected by a screening program. Although there is a theoretical basis for screening patients who have undergone an augmentation cystoplasty and a general clinical consensus that screening patients is prudent, there is as yet no evidence that screening programs are effective in the early detection of these often-aggressive tumours. Currently, cystoscopy is commonly performed every one or two years (Shaw and Lewis, 1999). However, patients with active transitional carcinoma of the bladder are usually screened every three to six months. When genetic changes are confirmed at the enterovesical anastomosis of patients who have undergone an enterocystoplasty it would seem reasonable to suggest that they should also be screened with a similar frequency.

Identifying a screening test for patients who have undergone an enterocystoplasty will be difficult, as the numbers of patients undergoing this procedure is relatively small compared with the numbers of patients with more common urological malignancies. Furthermore, the present incidence of clam

tumours remains low and the latency of these tumours is variable. However, there are a few tertiary centres where several hundred patients have undergone a clam cystoplasty. Therefore, a multicentre trial could be viable if enough evidence could be accrued to suggest that a particular method is of benefit in the early detection of clam tumours. However, further follow-up of the patients in this study is required before any particular method can be recommended for screening within the auspices of a multicentre trial.

Hopefully, ongoing research will increase the number of non-surgical treatments available to treat the overactive bladder. Consequently, there may be fewer patients who have an overactive bladder refractory to medical management and surgery may be required less often. Traditional anticholinergic drugs for the treatment of overactive bladder have been limited in their effectiveness. However, ongoing research has highlighted new medications which may be successful in alleviating symptoms without the need for surgery. Anticholinergic drugs currently available are inhibitors of M3 receptors (Chapter 1.1). However, the use of present anticholinergic drugs is limited as salivary glands and other tissues also contain M3 muscarinic receptors and treatment with these drugs is often associated with unpleasant side-effects. A truly bladder selective antimuscarinic drug with no associated dry mouth or central nervous system effects would be the ideal for treating overactive bladder (Yoshimura and Chancellor, 2002). Therefore, further studies are currently in progress to develop new M3 receptor antagonists which may be more effective and have an improved side-effect profile than traditional anticholinergic drugs. The M3 antagonists vamicamide (Yono *et al*, 2000) and darifenacin (Miyamae *et al*, 2003) have recently undergone clinical trials and may become available as alternative treatments for overactive bladder.

Alternative methods of administering anticholinergic drugs are under investigation and hopefully will reduce unwanted effects of treatment to a minimum. Intravesical oxybutinin for the management of the overactive bladder has been described (Lose and Nørgaard, 2001) but its use is limited as catheterisation is required three to four times daily. Transcutaneous delivery of oxybutinin may decrease the side effects of the drug whilst maintaining efficacy (Davila *et al.*, 2001) and transdermal patches containing oxybutinin are increasingly used for the treatment of overactive bladder (Cartwright and Cardozo, 2007). Research is currently ongoing to develop a long-lasting intravesical pump capable of releasing a precise drug quantity into the bladder over a period of time (Boone *et al.*, 2001). The concept involves a reservoir which can easily be inserted into the bladder and is not too small to be voided but is also not too large to cause bladder irritation or obstruction avoiding the need for repeated catheterisation.

Pharmacological therapies which will provide alternative treatments to the antimuscarinic drugs currently available for the management of overactive bladder are also being investigated. Direct bladder relaxation is induced by the activation of β -adrenergic receptors (Takeda *et al.*, 1999). The predominant β -receptor subtype in the bladder is the β_3 rather than the β_1 or β_2 receptor (Igawa *et al.*, 1999). Therefore, β_3 -adrenergic receptor subtype activation by directly relaxing bladder smooth muscle may be useful for treating overactive bladder (Woods *et al.*, 2001). Nonselective β -agonists have considerable systemic side effects including tachycardia and tremor that limit their clinical use. However, a highly selective β_3 agent has become available (with a thousand fold selectivity for activation of the β_3 receptor compared with the β_2 receptor with no measurable β_1 agonist activity)

which may be effective for the management of the overactive bladder (Rovner and Wein, 2003).

ATP acts on P2X and P2Y receptors in the bladder (Ralevic and Burnstock, 1998) and noradrenergic noncholinergic bladder contractions induced by adenosine triphosphate (ATP) increase in the bladder under certain pathological conditions including idiopathic urge incontinence and denervation (Sjögren et al, 1982). In particular, the expression of P2X2 receptors is significantly increased in patients with an overactive bladder while other P2X receptor subtypes are decreased (O'Reilly *et al.* 2002). Therefore, the purinergic pathway may provide a novel therapeutic approach for pharmacological treatment of an overactive bladder if receptor subtype specific agents become available.

Immunocytochemical studies indicate that bladder C-fibre afferent neurones contain various neuropeptides which are released into the bladder following noxious stimulation and may contribute to the inflammatory response by eliciting plasma extravasation, vasodilatation and alterations in bladder smooth-muscle activity (Lecci et al, 1993). Vanilloids such as capsaicin and resiniferatoxin (RTX) activate nociceptive sensory nerve fibres through an ion channel known as vanilloid receptor subtype 1 (Caterina *et al.*, 1997). These receptors are predominantly located on C-fibre bladder afferent nerve fibres and activating the receptors initially excites and subsequently desensitises C-fibres (Chancellor and de Groat, 1999). Intravesical capsaicin significantly decreases bladder hyperactivity in patients with spinal cord injury (Wiart *et al.*, 1998) and idiopathic detrusor instability (DeRidder *et al.*, 1997) and its effect on clinical and urodynamic parameters may be prolonged (greater than one year) in selected patients (Chancellor and de Groat, 1999). However, intravesical

capsaicin initially causes stimulation of C-fibres and release of neurotransmitters resulting in severe discomfort or pain. RTX is approximately one thousand times hotter than capsaicin based on the Scoville heat scale and is therefore a much more potent sensory antagonist than capsaicin. Administration of RTX, which is also a vanilloid receptor agonist, results in desensitisation but lacks the initial potent neuronal excitatory effect of capsaicin and is therefore associated with less discomfort. RTX has been used to successfully treat patients with neurological and idiopathic overactive bladders, the improvement obtained lasting up to three months (Cruz *et al*, 1997a, Cruz *et al*, 1997b). Furthermore, RTX has also been reported to be helpful in patients in whom capsaicin was ineffective (Lazzeri *et al*, 1998). However, RTX and capsaicin are currently experimental drugs and further clinical trials are required to evaluate the efficacy and safety of these drugs.

Currently there are no drugs in clinical use for the management of the overactive bladder that act on the central nervous system. However, recent experimental studies have suggested potential targets for central nervous system acting drugs in the brain and spinal cord (Yoshimura and Chancellor, 2002). The sphincter motor nuclei in the brainstem receive input from 5-hydroxytryptamine (serotonin) containing neurones from the raphe nuclei in the caudal brainstem. Activity in the serotonergic pathway generally enhances urine storage via stimulation of the vesical sympathetic reflex pathway and inhibition of the parasympathetic voiding pathway (Sharma *et al*, 2000). Duloxetine, a selective serotonin and noradrenaline re-uptake inhibitor, has been shown to significantly increase bladder capacity and sphincter tone in cats without interfering with the normal voiding cycle (Thor and Katofiasc, 1995). Duloxetine initially was thought to be effective in the management of stress incontinence (Dmochowski *et al*, 2003) and clinical trials are

in progress to assess its role in the management of the overactive bladder (Rovner and Wein, 2003). However, there are now considerable doubts to the efficacy of duloxetine in treating stress incontinence and the National Institute for clinical excellence (NICE) has suggested that duloxetine should be used neither as first nor second line for treating women with stress incontinence. Rather, duloxetine should be reserved for the treatment of women who are not suitable for surgery (Duckett, 2008).

In the central nervous system dopaminergic receptors exert inhibitory and facilitatory effects on the micturition reflex. D1 and D5 receptors are inhibitory whereas D2, D3 and D4 receptors promote the micturition reflex (Kontani *et al*, 1990). The D1 agonists pergolide and SKF 38393 have been shown to suppress the detrusor contractions of drug-induced parkinsonian monkeys with overactive bladders (Yoshimura *et al*, 1993, Yoshimura *et al*, 1998). Possibly therefore, drugs acting on the dopaminergic system may be useful in the management of the overactive bladder.

γ -Aminobutyrate (GABA) is an inhibitory neurotransmitter in the brain and spinal cord and acts on GABA-A and GABA-B receptors. Injection of either GABA or the GABA receptor agonist muscimol into the pontine micturition centre of cats suppresses reflex bladder activity and increases the volume threshold for inducing voiding (Mallory *et al*, 1991), effects which are reversed by the GABA-A receptor antagonist bicuculline. In spinal cord injured patients detrusor hyperreflexia has been treated with intrathecal infusion of the GABA-B agonist baclofen (Ochs, 1993). These observations suggest that drugs which stimulate GABAergic receptors may be

useful in treating patients with an overactive bladder and further studies are required to substantiate this hypothesis.

Botulinum toxin is the most potent biological toxin known and acts by inhibiting acetylcholine (ACh) release at presynaptic cholinergic junctions. Injection of botulinum results in muscle atrophy and regionally decreased muscle contractility at the site of administration (Duchen, 1970). The effects of botulinum are reversible and axons resprout in three to six months (Borodic *et al*, 1990). Botulinum A toxin has been used to elicit a reversible chemical sphincterotomy in patients with spinal cord damage and detrusor-sphincter dyssynergia, thereby avoiding a surgical sphincterotomy with its attendant risks of bleeding, stricture and fistula (Petit *et al*, 1998). Recently, patients with a spinal cord injury and an overactive bladder have been treated with multiple intravesical injections of botulinum toxin (Schurch *et al*, 2000). Seventeen out of nineteen patients treated with intravesical botulinum toxin were continent at six weeks but all patients were incontinent by thirty-six weeks (Schurch *et al*, 2000). Preliminary studies have also been described where multiple intravesical botulinum injections have been performed in paediatric patients with myelomeningocele and an overactive bladder (Riccabona *et al*, 2004), women with idiopathic detrusor instability (Dykstra *et al*, 2003a) and in a patient with multiple sclerosis and an overactive bladder (Dykstra *et al*, 2003b). Recent randomised controlled trials have reported a significant improvement with the use of botulinum toxin in patients with a neurogenic overactive bladder with continence rates of between forty and eighty per cent (Karsenty *et al*, 2008). There have also been several studies using botulinum toxin in patients with an idiopathic overactive bladder. Efficacy has been demonstrated in the vast majority of reported series and the side effects of intravesical botulinum toxin appear to be minimal and short lived

(Patel *et al.*, 2006). However, a recent Cochrane Review has concluded that currently there is not enough controlled trial data on the benefits and safety of intravesical botulinum toxin in patients with an idiopathic overactive bladder compared with other interventions, or with placebo (Duthie *et al.*, 2007). This review also stated that presently the optimal dose of botulinum toxin for efficacy and safety has not yet been found (Duthie *et al.*, 2007). Therefore, large randomised controlled trials are required to determine the precise role of intravesical botulinum toxin.

Gene therapy, which selectively alters gene expression in cells with genetic abnormalities requires the transfer of genetic material into cells by viral and nonviral (liposomes and direct injection of naked DNA) delivery systems (Siemens *et al.*, 2003). Intravesical injection of vectors ensures efficient delivery of the gene product to the bladder and avoids systemic complications (Christ, 2004). Possible gene therapy strategies for the overactive bladder include suppression of bladder muscle activity or inhibition of the neural pathways that elicit the micturition reflex. There is evidence to suggest that altered electrical properties of myocytes may contribute to the aetiology of overactive bladder (Turner and Brading, 1997) and potassium channels are thought to play an important role in bladder overactivity (Andersson, 1997). Calcium-sensitive potassium channels are antagonists for transmembrane calcium flux and are therefore important modulators of detrusor smooth muscle tone (Imaizumi *et al.*, 1998). Increased expression of calcium-sensitive potassium channels is thought to diminish smooth muscle tone but has little effect on the ability of the bladder to empty during a contraction (Christ, 2004). Preliminary studies using potassium channel gene therapy have shown promise for the treatment of the overactive bladder. Thus, intravesical inoculation of naked DNA for the calcium activated potassium channel gene suppressed bladder contraction in rats with bladder

outflow obstruction and overactive bladders (Christ *et al*, 2001). The continuing evolution of gene transfer vectors and gene delivery technologies is expected to further enhance the selectivity, efficacy and duration of gene therapy making it a viable potential treatment option for the overactive bladder.

Further work is required before any of the novel pharmacological or genetic therapies discussed in previous sections become accepted as a recognised treatment for the overactive bladder. However, because of the anticipated increase in the number of drugs available for the management of the overactive bladder, there is an increased interest in pharmacogenomics (Yoshimura and Chancellor, 2002). Pharmacogenomics is the study of the genetic makeup of individuals using micro-array gene chip technology (Solinas-Toldo *et al*, 1997) and may be used to determine an individual's drug metabolism profile, receptor profile and allergy risk. These factors can then be screened against a list of possible medications and therapy tailored for individual patients. In the future, it is likely that fewer operations will be performed to control incontinence due to the increased number of non-surgical therapies becoming available for the management of overactive bladder. It is unlikely that this reduction in surgical workload will have any appreciable effect on the workload of most urology centres; however this may markedly reduce the number of operations performed in specialist reconstructive units. However, even with an expansion in the range of non-surgical therapies, some patients may still experience an unacceptable quality of life. In patients where symptoms are refractory to medical management or who are not motivated to persevere with non-pharmacological techniques, surgery may be the only option.

Until recently, patients with an overactive bladder refractory to pharmacological management were frequently offered a clam ileocystoplasty. However, concerns about the malignant potential of enterocystoplasties have a firm theoretical basis (Chapter 1.3.3) and the results of the present study clearly indicate that genomic instability is present at the enterovesical anastomosis in patients who have undergone an ileocystoplasty (Chapter 3). Incorporating ileum into the bladder effectively provides symptomatic control of an often benign, if socially unacceptable condition such as idiopathic detrusor instability. However, if bladder augmentation leads in the long term to the development of a tumour which would not have otherwise developed, then we are doing our patients a disservice. The surgical management of patients with an intractable overactive bladder is therefore problematic.

Surgical techniques such as phenolisation (Blackford *et al*, 1984) and bladder transection (Mundy, 1983), widely used in the past for the management of a drug resistant overactive bladder, have now been abandoned because of high complication rates and a limited efficacy (Rosenbaum *et al*, 1990). Endoscopic bladder transection has also been used in the past as a treatment for overactive bladder but is no longer used as the operation only produces only a transient symptomatic relief in a few of those patients who have failed to respond to pharmacological manipulations (Hasan *et al*, 1995).

Malignant transformation following an ileocystoplasty would appear to depend on the apposition of transitional mucosa adjacent to bowel epithelium. Therefore, surgical techniques, which do not create a bladder-bowel interface, may avoid the risk of developing a life-threatening tumour. A small proportion of patients,

primarily those with a neuropathic bladder with significant upper tract dilatation and poorly functioning kidneys would be candidates for ureterocystoplasty (Bellinger, 1993). Several studies have suggested that ureterocystoplasty provides good symptomatic control for the management of the overactive bladder without the risk of late malignant change observed following non-urothelial augmentation (Churchill *et al*, 1993, Perovic *et al*, 2000). Ureterocystoplasty is not suitable for the majority of patients with an overactive bladder because of limitations in the amount of urothelium available for augmentation. However in experimental animals ureteric expansion has been achieved by placing a tissue expander in the lumen of the ureter to generate urothelium for use in bladder augmentation (Ikeguchi *et al*, 1998). Therefore, ureteric expansion may be a technique that could be used to facilitate urothelial augmentation of the bladder in a larger number of patients than is currently possible.

Detrusor myectomy is another surgical procedure where the bladder is augmented without using bowel (Cartwright and Snow, 1989). In brief, the dome of the bladder is exposed via a transperitoneal incision, the detrusor muscle excised from the upper half of the bladder and the denuded urothelium is completely covered with a layer of omentum. Detrusor myectomy is reasonably effective in controlling detrusor overactivity in neuropathic patients and those with detrusor overactivity from childhood (Stephenson *et al*, 2001). However, the technique appears to be less successful in patients with idiopathic detrusor instability who numerically form the largest group of patients who present with detrusor overactivity (Swami *et al*, 1998, Stephenson *et al*, 2001).

Seromuscular enterocystoplasty is a procedure that combines clam enterocystoplasty with detrusor myectomy (Gonzalez *et al*, 1995). In brief, the bladder detrusor muscle is excised and the urothelium covered with a demucosalised bowel segment to form a compliant bladder that is lined with urothelium supported by muscle. Results from studies in man and experimental animals indicate that seromuscular enterocystoplasty improves bladder compliance and capacity (Gonzalez *et al*, 1995). However, although seromuscular enterocystoplasty shows promise, currently, very few patients have undergone this procedure and hence, follow-up is limited (Rink, 1999). In particular, the long-term effects of lining an intestinal segment with urothelium are unknown. Intestinal mucosal regrowth is a concern and biopsies from patients who have undergone seromuscular colocystoplasty suggest that the segment of bowel comprises mixed colonic mucosa and urothelial lining (Gonzalez *et al*, 1995). It has been suggested therefore that removal of the muscularis mucosa and inner portion of submucosa of the bowel is imperative to prevent the regrowth of enteric mucosa (Dewan *et al*, 1997). Recently, a similar procedure to seromuscular enterocystoplasty has been described where urothelium-preserving augmentation cystoplasty is covered with a peritoneal flap (Oge *et al*, 2000). However, experience with this procedure is limited, particularly in terms of follow-up.

In the future, tissue-engineering techniques offer the possibility of bladder augmentation with urothelium, thereby avoiding the need for an enterovesical anastomosis. Currently, *in-vitro* and *in-vivo* tissue-engineering technologies are being used to produce regenerated tissue grafts:

In-vitro tissue-engineering uses biodegradable membranes that are seeded with urothelial cultured cells that have been established from biopsies from the host (Atala *et al*, 1992). The urothelial cells are grown in culture until the desired numbers have been obtained and it is possible to expand an urothelial strain from a single sample of urothelium that initially covers 1 cm² to one covering a surface area of 4202 m² within eight weeks (Cilento *et al*, 1994). This composite graft is then placed back in the host for the continuation of the regenerative process (Atala *et al*, 1993). *In-vitro* tissue-engineered bladders have been successfully used to augment the bladders of dogs that had undergone a partial cystectomy (Yoo *et al*, 1998) but as yet, there are no published reports of this procedure in humans.

In-vivo tissue-engineering involves the use of a biodegradable scaffold that the host bladder can use to remodel and regenerate tissue and is based on the natural ability of the cell to heal and regenerate. Small-intestinal submucosa (SIS) is an acellular, collagenous membrane harvested from porcine small intestine after the mucosa, serosa and muscularis have been removed. SIS differs from other graft material in containing functional growth factors that are probably vital to the regenerative process (Voytik-Harbin *et al*, 1997). SIS has been successfully used for bladder augmentation in both rats (Kropp *et al*, 1995) and dogs (Kropp *et al*, 1996). SIS regenerated bladders display contractile properties and nerve regeneration similar to that exhibited by non-augmented bladders (Vaught *et al*, 1996) and is currently in clinical use as a urethral sling for the treatment of stress incontinence (Colvert *et al*, 2002).

At present, there is evidence to suggest that regeneration of bladder can be achieved using currently available tissue-engineering techniques and the clinical

potential of these technologies shows a great deal of promise in man for the management of detrusor instability. However, caution should be exercised, as it is not yet clear whether bladder augmentation with tissue-engineering techniques would abolish unwanted detrusor contractions in those patients with an overactive bladder. However, if augmenting the human bladder with tissue-engineered transitional epithelium is effective in treating the overactive bladder and does not predispose patients to a risk of tumour formation, the procedure would be preferable to a conventional augmentation cystoplasty

Presently, clam enterocystoplasty is still indicated in a small group of patients, particularly those with neuropathic pathology, in order to prevent or arrest the development of hydronephrosis and deterioration in renal function secondary to an overactive bladder. However, as it is the bladder-bowel interface that is at risk of malignant transformation following an enterocystoplasty, a substitution cystoplasty may be a safer long term procedure since the urothelial bowel anastomosis is limited to the the two ureteral anastomoses with possibly a urethral anastomosis. Patients undergoing a clam cystoplasty or a substitution cystoplasty should be informed of the risks of long-term malignant transformation and the need for regular tumour surveillance before undergoing the procedure.

The incidence of carcinoma following enterocystoplasty although small, increases with time and there is a requirement to develop techniques to identify those patients who will develop a life-threatening tumour. The ileovesical anastomosis within a clam ileocystoplasty is inherently genetically unstable resulting in an increased rate of mutagenesis of the epithelium. In the present study, chromosomal abnormalities at the bladder-bowel interface were detected in all of the patients

studied using FISH. In particular, aneuploidy of chromosome 18 was frequently present and appears to be a good marker of genetic instability at the ileovesical anastomosis. P53 mutations identified by the RSM assay also appeared to be good markers of genomic instability at the ileovesical anastomosis of patients who have undergone a clam ileocystoplasty. However, the early chromosomal changes at the ileovesical anastomosis following an ileocystoplasty identified using FISH and the RSM assay in the present study are only markers of instability and are not necessarily the specific genetic changes that will initiate tumour formation. Further longitudinal studies are required to establish more precisely which genetic changes are involved in the initiation, growth and progression of cancers arising within augmentation cystoplasties. However, both FISH and the RSM assay show promise as screening techniques that may prove useful in identifying those patients most at risk of developing a life-threatening tumour.

6. Conclusions.

The incidence of carcinoma within an enterocystoplasty is small but increases with time. Since cancers occurring within clam ileocystoplasties are highly aggressive and have developed rapidly by the time they are diagnosed, a method that predicts tumour formation would be of obvious benefit in the management of these patients. The results of the present study indicate that chromosomal changes and p53 mutations, suggestive of genetic instability are present in morphologically normal tissue obtained from patients with an enterocystoplasty.

In the present study, fluorescent *in-situ* hybridisation (FISH) detected chromosomal abnormalities at the bladder-bowel interface, but not elsewhere in the native bladder, in all of the patients studied. These observations suggest that the enterovesical anastomosis is inherently genetically unstable resulting in an increased rate of mutagenesis of the epithelium. However, the chromosomal abnormalities identified in the present study do not identify those patients who have undergone an enterocystoplasty who are at an immediate risk of developing life-threatening tumours. Nevertheless, the high frequency of aneuploidy at the anastomosis suggests that these chromosomal changes may precede the formation of invasive cancer. In particular, aneuploidy of chromosome 18 was frequently present and appears to be a good marker of genetic instability at the ileovesical anastomosis. However, further work is required to determine whether chromosome 18 aneuploidy is useful as a marker to predict cancer formation or alternatively if aneuploidy of chromosomes 8 or 9 will prove to be more useful markers.

In the present study a large number of polyploid cells were detected in tissue obtained from a clam cancer using FISH. These observations provide further evidence of the aggressive nature of clam tumours since polyploid neoplasms are known to have an extremely poor prognosis. Therefore, identification of polysomic cells at the enterovesical anastomosis of patients who have undergone an enterocystoplasty would be a cause for concern and may also prove to be a prognostic marker for cancer formation. Further investigation of the genetic abnormalities arising in clam tumours and biopsies in augmentation cystoplasties may suggest further cytogenetic changes or mutations that might identify those patients with an enterocystoplasty who will go on to develop tumours.

The results of the present study have shown that p53 mutations indicative of genetic instability occur at the ileovesical anastomosis in patients who have undergone a clam ileocystoplasty. Several of the detected mutations were GC → AT transitions, resembling those induced by reactive oxygen species and N-nitrosamines, suggesting a possible role of these agents in the aetiology of carcinoma formation in clam ileocystoplasty. It is not clear whether patients displaying p53 changes as early mutational events progress to cancer more rapidly than patients without p53 mutations. However, p53 mutations have been shown to be markers of anastomotic instability in clam ileocystoplasties of some patients, irrespective of whether the specific changes identified lead to a malignant tumour. Therefore, p53 mutations identified by the restriction site mutation (RSM) assay at the enterovesical anastomosis of patients who have undergone a clam ileocystoplasty may prove useful as biomarkers for instability and cancer risk.

The results of this study suggest detection of chromosomal losses and gains may be a useful marker for tumour formation but this suggestion requires further investigation. The early chromosomal changes at the ileovesical anastomosis following an ileocystoplasty identified using FISH and the RSM assay in the present study are only markers of genetic instability and are not necessarily the specific genetic changes that will initiate tumour formation. Further longitudinal studies are required to establish more precisely which genetic changes are involved in the initiation, growth and progression of cancers arising within augmentation cystoplasties. However, both FISH and the RSM assay show promise as screening techniques that may prove useful in identifying those patients most at risk of developing a life-threatening tumour.

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Appendices

Appendix 1. Suggestions for future research.

The results of the present study indicate that chromosomal aneuploidy and p53 mutations, indicative of genetic instability, occur at the ileovesical anastomosis in patients who have undergone a clam ileocystoplasty. Long-term, longitudinal follow-up of patients with genetic abnormalities in anastomotic biopsies is required to establish whether genetic abnormalities persist, accumulate and become more prevalent with time and increase the risk of cancer formation. Furthermore, multiple biopsies from individual patients taken from different locations along the anastomosis could be examined to determine whether any observed changes are confined to a localised area of the anastomosis or are present throughout its length.

The results of the present study suggest that fluorescent *in-situ* hybridisation (FISH) is a sensitive technique for identifying chromosomal changes in biopsies taken from the enterovesical anastomosis in patients who have undergone a clam ileocystoplasty. However, FISH of exfoliated bladder cells has been reported to be more sensitive than conventional urine cytology for the detection of transitional cell carcinoma of the bladder (Halling *et al*, 2000). Obtaining biopsies from an enterocystoplasty is an invasive procedure associated with a low but significant incidence of complications. In contrast, urine cytology is safe, non-invasive and can be performed as an outpatient procedure. Further work is required to determine whether the same abnormalities detected by FISH on touch preparations obtained from bladder biopsies can be detected in exfoliated cells in the urine of patients who have undergone an enterocystoplasty. However, further prospective, longitudinal studies would be required to determine whether these changes are associated with a higher risk of cancer formation.

The present study has looked for changes in the p53 genes and at aneuploidy in chromosome 8, 9, and 18 in biopsies from patients with an enterocystoplasty. However, it is possible that other genes or aneuploidy of different chromosomes may be more effective than those occurring in chromosomes 8, 9 and 18 in predicting those patients with an enterocystoplasty who will develop a tumour. Further investigation of the genetic abnormalities arising in clam tumours and biopsies in augmentation cystoplasties may suggest alternative cytogenetic changes or mutations in clam tumours that may help identify those patients with an enterocystoplasty who will go on to develop tumours. As other clam cancers occur, where possible tumour material should be obtained for genetic analysis.

There is evidence from epidemiological studies to suggest that non-steroidal anti-inflammatory drugs (NSAIDs) such as aspirin may decrease the incidence of certain cancers by inhibiting cyclo-oxygenase 2 (Wang and Dubois, 2006). Further cohort studies are required to establish whether regular consumption of NSAIDs is associated with fewer genetic abnormalities and reduces the risk of tumour formation in patients with an enterocystoplasties than control patients who have undergone the same procedure. However, a prospective randomised controlled, longitudinal trial would be required to determine whether regular consumption of NSAIDS is associated with a lower incidence of genetic abnormalities and cancer formation. In order for the trial to be sufficiently powered to detect statistically significant differences between the groups, a large number of patients, possibly from more than one centre, would need to be recruited into the trial.

If a genetic technique is identified that could reliably predict the formation of cancer within an enterocystoplasty, the optimal screening frequency would need to be determined. Further, trials would be necessary to establish that screening was effective in reducing the mortality from clam cancers. Augmentation cystoplasty is carried out in a small number of tertiary centres and a multicentre trial could be arranged between centres in order to enrol a sufficient number of patients into a trial.

Appendix 2. Publications derived from the work in this thesis:

Abstract: Ivil KD, Parry EM, Parry JM, Stephenson TP. Fluorescence in-situ hybridisation on biopsies from clam ileocystoplasties.

Journal of Urology 2003; 169 (Suppl 4): 63-64.

(Presented at the American Urological Association 98th Annual Meeting in Chicago).

Abstract: Ivil KD, Jenkins GJ, Parry EM, Parry JM, Stephenson TP. Identification of early p53 mutations in clam ileocystoplasties using the restriction site mutation assay.

Journal of Urology 2003; 169 (Suppl 4): 139.

(Presented at the American Urological Association 98th Annual Meeting in Chicago).

Ivil KD, Doak SH, Jenkins SA, Parry EM, Kynaston HG, Parry JM, Stephenson TP. Fluorescence in-situ hybridisation on biopsies from clam ileocystoplasties and on a clam cancer.

British Journal of Cancer 2006; 94 (6): 891-895.

Ivil KD, Jenkins SA, Doak SH, Hawizy AM, Kynaston HG, Parry EM, Jenkins JS, Parry JM, Stephenson TP. Identification of early P53 mutations in clam ileocystoplasties using the restriction site mutation (RSM) assay.

Accepted for publication in Urology, 29th June 2007.

Materials and Methods.

Patient population.

DNA obtained from biopsies from thirty-eight patients who had previously undergone a clam ileocystoplasty was used for RSM analysis. Clam ileocystoplasty was performed for the treatment of a neuropathic bladder in twenty patients and for the management of an overactive non-neuropathic bladder in seventeen patients (ten of the latter patients had a congenitally unstable bladder). One further patient underwent a clam ileocystoplasty for the treatment of tuberculous cystitis. Previous colorectal cancer, premalignant lesions of the colon or a strong family history of colorectal cancer is considered a contraindication to clam ileocystoplasty; such patients were not considered for augmentation cystoplasty. Patients requiring chronic catheterisation for the management of infection and/or persistent difficulties with voiding were excluded from the study. The mean age of patients at the time of their biopsy was 35.7 years (median 30, range 18 to 65) and the average time between clam ileocystoplasty and the present study was 12.4 years (median 11.5, range 4 to 41).

Collection of biopsy samples.

The study was approved by the Regional Ethics Committee and written informed consent was obtained from all patients. It is standard practice in our unit that patients with a clam enterocystoplasty are followed-up yearly by rigid cystoscopy and biopsy of the urothelium at the enterovesical anastomosis. In addition to the usual biopsy for histology, a biopsy was taken from the enterovesical anastomosis and from the native bladder remnant at least 3 cm from the enterovesical anastomosis (control specimen) and the biopsies stored dry in a freezer at -20°C.

DNA extraction.

The biopsies were subjected to high-salt DNA extraction (Stratagene, Cambridge, UK). The quantity and purity of the DNA was assessed by spectrophotometry (260/280 nm) and by six percent polyacrylamide gel electrophoresis, the concentration adjusted to 100 ng/μl and stored at –20°C.

RSM analysis: The DNA extracted from anastomotic and control biopsies from the native bladder remnant were analysed for mutations occurring in restriction sites of the *P53* gene. The RSM assay was used to study five known hotspots using the restriction enzymes (Promega Corporation, Southampton, UK) Hha I (codon 175), Taq I (codon 213), Hae III (codon 249/250) and Msp I (codons 248 and 282). *P53* mutational events were confirmed by sequencing the undigested PCR products identified by RSM analysis as previously described⁷.

Results.

The incidence of RSM mutations at the enterovesical anastomosis (7 of the 38 patients) was significantly higher ($p = 0.0057$ Fishers Exact test) than in urothelial tissue from the native bladder distant to (>3 cm) to the anastomosis. Age at operation, age at biopsy and the latent period between the time of operation and the time of study was not significantly different between patients with P53 mutations and those without (Table 1). Similarly, there was no significant difference in the incidence of P53 mutations at the enterovesical anastomosis between males and females ($p = 0.6546$; Fisher's exact test) or between patients who underwent a clam enterocystoplasty for a neuropathic bladder and those with a non-neuropathic bladder ($p = 0.6463$; Fisher's exact test). Logistic regression analysis indicated that age-related variables, gender or the indication for clam ileocystoplasty did not independently influence the risk of P53 mutations at the enterovesical anastomosis (Table 2). Of the 7 mutations detected 1 patient had a mutation at codon 213, three a mutation of codon 248 and three a mutation of codon 250 (Table 3). Six of the 7 mutations detected in this study were with exon 7, specifically codons 248 and 250, suggesting that mutations were preferentially induced in this region. The restriction site for the endonuclease Hae III covers both codons 249 and 250 and was chosen in the present study primarily to investigate changes at codon 249 of the *P53* tumour suppressor gene, a recognised hotspot for mutations. However, in this study, 3 of the 7 mutations were found at codon 250.

Sequencing was used to characterise the mutations identified. In one sample sequencing was unsuccessful due to technical difficulties because of the poor quality of the PCR product. Of the 6 mutations characterised, 4 (67%) were GC→AT transitions, three of them at CpG sites. Five of the

6 mutations contained just one point mutation. However, a CGG→CGC transversion identified at codon 248 was part of a complex mutation where several point mutations were present.

No histological evidence of dysplasia or malignancy was observed in biopsies from the enterovesical anastomosis or native bowel remnant in any of the 38 patients.

Discussion.

The results of the present study indicate that rare *P53* mutations, detected by RSM analysis, occur at the ileovesical anastomosis in patients with a clam ileocystoplasty confirming previous observations suggesting that the enterovesical anastomosis is genetically unstable^{11, 12}. Since genetic instability has been shown to progress to cancer formation in a variety of tumour types¹³ the results of this study provide further evidence that patients with an enterocystoplasty may be susceptible to an increased risk of tumour development. Furthermore, the *P53* mutations occurred in endoscopically and histologically normal tissue indicating that mutation occurs prior to morphological and histological abnormalities.

In two patients a silent mutation (i.e. a nucleotide is altered to produce a synonymous codon without amino acid change) was detected. Since the *P53* sequence was not altered, cells with these mutations do not undergo clonal expansion. However, silent mutations can accumulate with time due to increased mutagen exposure and approximately 10 *P53* molecules with identical mutations are required to obtain an identifiable PCR product with the RSM assay^{7, 14}. The majority of silent mutations in the *P53* gene are randomly selected, neutral and usually occur in the presence of a 'driver' mutation which may be in *P53* or in another gene that is part of the *P53* cascade¹⁵. It is unlikely therefore that the silent *P53* mutations observed at the enterocystoplasty of patients with an ileocystoplasty promote tumourogenesis. However, mutations identified by RSM analysis are indicative of genomic instability at the enterovesical anastomosis, and therefore may be useful in identifying those patients at an increased risk of developing a life-threatening tumour.

Of the 7 mutations detected in this study 6 were within *P53* exon 7: 3 in codon 248 and 3 within

250. The restriction site for the endonuclease Hae III covers both codons 249 and 250 and was chosen primarily to investigate changes at codon 249 of the *P53* tumour suppressor gene, a recognised hotspot for mutations. It is of interest that mutations of codon 250 have been only occasionally reported (TP53 Mutation Database; [www. iarc.fr](http://www.iarc.fr)). However, in this study 3 of the 7 mutations were found at codon 250. Exon 7 is a highly conserved region and is located within the sequence specific binding domain of the *P53* protein¹⁶. The mutations identified in this study may therefore prevent the protein from binding to its consensus sequence, thus reducing or even abolishing *P53* mediated repair following environmental stresses. The mutations at codons 248 & 250 may therefore predispose to a further accumulation of genetic alterations that aid neoplastic progression. Furthermore, 6 out of the 7 mutations were identified at codons 248 & 250 suggesting that mutations were preferentially induced in this region which may therefore represent *P53* mutational hotspots specific to the enterovesical anastomosis.

Nitrosamines are potent carcinogens and elevated urinary nitrosamine levels have been reported in patients with an enterocystoplasty¹⁷, particularly those with concomitant bacteriuria and chronic inflammatory changes in the cystoplasty¹⁸. These observations suggest that patients with an inflamed cystoplasty and an accompanying infection may be at high risk from the carcinogenic effect of urinary nitrosamines. The type of *P53* mutation identified at the anastomosis following clam ileocystoplasty in the present study may implicate certain carcinogenic agents. CpG sites are those where a cytosine nucleotide lies adjacent to a guanine nucleotide joined by the sugar-phosphate backbone in the DNA helix. CpG sites are known to be susceptible to spontaneous deamination of methylated cytosine leading to the conversion of methyl-cytosine to thymine and this process is thought to be enhanced by exposure to mutagens or inflammation¹⁹. Many types of oxygen radical are produced by inflammation

and these generate up to twenty different types of DNA adducts²⁰. Of the six mutations characterised by sequencing, four (67%) were GC → AT transitions, three of them at CpG sites. GC → AT transitions at CpG sites are typical findings after either Nitrosamine exposure²¹ or after oxidative damage to DNA¹⁹. A CGG → CGC transversion identified at codon 248 was part of a complex mutation and such events are commonly described after nitrosamine exposure²². Hence, it is possible that reactive oxygen species and N-nitrosamines may have contributed to *P53* mutations detected at the ileovesical anastomosis of patients following a clam enterocystoplasty in the present study, a suggestion supported by preliminary observations indicating the presence of free radicals in the urine of such patients¹¹. Following the loss of *P53* mediated protection, the continued presence of these carcinogenic agents may consequently trigger the accumulation of further genetic aberrations that are necessary for further malignant transformation. The *P53* mutation rate at the ileovesical anastomosis in the present study (18%) is markedly higher than the risk of tumour formation (0.9%) within an ileocystoplasty³. Possibly therefore, although *P53* mutations may increase the risk for tumour formation within an ileocystoplasty, only a small proportion will acquire the further genetic alterations required of tumour formation. However, the frequency of mutations in the *P53* gene in ileocystoplasty tumours is unknown and needs to be established in order to determine the risk between *P53* mutations and tumourogenesis.

The RSM assay is a new technology that has recently been used to detect early *P53* mutations in several premalignant conditions^{7, 8, 9, 19}. Further long-term longitudinal follow up studies are required to confirm if the mutations detected at the enterovesical anastomosis in the present study are associated with subsequent cancer formation. Therefore, although the *P53* gene plays a pivotal role in tumour suppression and loss of *P53* function may well predispose to tumour formation, it is not yet clear

whether patients with early *P53* mutational events progress to cancer more rapidly than patients without these mutations. In the present study, the *P53* changes detected at the anastomosis following clam ileocystoplasty are indicative of genomic instability but it is not possible to link these changes to tumour formation. Therefore, it is possible that the RSM assay may prove useful in identifying those patients who are at an increased risk of developing invasive tumours. However, prospective, blinded longitudinal studies are required however to determine whether predetermined *P53* mutations predict the development of tumours with an augmentation ileocystoplasty.

Conclusions.

The results of the present study indicate that *P53* mutations, indicative of genetic instability occur at the ileovesical anastomosis in patients who have undergone a clam ileocystoplasty. Long-term follow up of patients with and without *P53* mutations at the enterovesical anastomosis are required to establish whether these early *P53* mutations are associated with an increased cancer risk. If this hypothesis is confirmed in further studies, detection of early *P53* mutations using RSM may be useful for identifying those patients most at risk of developing a life-threatening tumour following a clam enterocystoplasty.

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Table 1: Demographic details of patients with or without P53 mutations at the enterovesical anastomosis following an enterocystoplasty

	P53 Mutations Present	No. P53 Mutations	P-value
Age at operation (y)			
All patients	17 (12-48)	18.5 (9-54)	0.658
Males	15,17, 48	17.5 (11-41)	
Females	12,12,18,20	22 (9-54)	
Neuropathic	15 (12-48)	17.5 (12-54)	0.881
Congenital	18	18 (9-49)	
Age at biopsy (y)			
All patients	28 (21-61)	33 (18-65)	0.634
Males	21, 28, 58	28 (22-63)	
Females	22,23,31,61	35 (18-65)	
Neuropathic	23 (21-58)	28.5 (22-65)	0.688
Congenital	31	25 (18-61)	
Latent period (months)			
All patients	11.5 (6-41)	12 (4-43)	0.713
Males	6,10,14	11.5 (8-43)	
Females	10,11,13,41	12 (4-18)	
Neuropathic	10 (6-16)	10.5 (4-18)	0.821
Congenital	13	11.5 (8-14)	

The results are expressed as the median (range). Individual values are given when N<6. The statistical significance of differences between the groups was evaluated by the Mann Whitney U Test. No formal comparisons were made when N<5.

Table 2: Logistic regression analysis of demographic variables on the incidence of P53 mutations at the enterovesical anastomosis following an enterocystoplasty

	Wald statistic	$G\chi^2$ (likelihood ratio)	Significance
Age at operation	0.376	1.625	0.540
Age at biopsy	0.423	0.589	0.515
Latent period between time of operation and biopsy	0.445	1.732	0.505
Gender	0.000	1.007	0.994
Indication for operation	0.117	1.252	0.732

Table 3. P53 mutations identified by RSM analysis in patients who have undergone clam ileocystoplasty.

Exon	Codon	Mutation	Amino-acid change
6	213	CGA→CGG	Silent mutation (arginine)
7	248	CGG→CGC	Silent mutation (arginine)
7	248	CGG→TGG (within complex mutation)	Arginine→tryptophan
7	248	Unable to sequence RSM product	
7	250	CCC→TCC	Proline→serine
7	250	CCC→CTC	Proline→leucine
7	250	CCC→TCC	Proline→serine

